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VOLUME II

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Plant Pathology

An Advanced Treatise

Edited by

J. G. HORSFALL AND A. E. DIMOND

The Connecticut Agricultural Experiment Station
New Haven, Connecticut

VOLUME II

The Pathogen

1960
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CHAPTER 1

Prologue

The Pathogen: the Concept of Causality

J. G. HORSFALL AND A. E. DIMOND

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I. INTRODUCTION

In Volume I we discussed the diseased plant and what disease is. In Volume II we shall discuss the antecedents of disease, how disease originates, in short what causes disease. Cause is an abstract term, the derivation of which has been a slow and painful business in plant pathology. Even yet there are murky pockets in the concept. The murkiness has been worsened recently by the rise of a new and unfamiliar term, *to incite.*

Other difficulties confuse the issue. One of these is the fallacy of confusing parasite with pathogen, parasitism with pathogenism. Then we have two other loosely used words, pathogenicity and pathogenesis. A brief excursion or two into etymology should serve to aid in understanding the concepts.

The commonest booby trap in using these terms is to state very clearly that pathogenesis is the chain of causality in disease, that parasitism is the ability of an organism to procure food from the host, and then proceed to use them almost interchangeably.

II. What Is the Pathogen?

The significance of the word pathogen can be worked out from its two parts. It derives from two Greek words, *pathos* meaning suffering, and *genesis* origin. Genesis in turn derives from the Greek word, *gignomai*, to be born. An agency that gives birth to or generates suffering is a pathogen.

As we have said in the definition of disease, a pathogen is constantly associated with disease in the typical case.

A pathogen is an irritant. It seems to us that the word irritant should enjoy a wider usage in plant pathology. Its use in our language of plant pathology will serve to suggest the more or less continuous operation of the pathogen. B. M. Duggar, in a perspicacious analysis of plant pathology for Volume I of *Phytopathology* (1911), proposed that "The basis of the whole science of plant pathology rests upon the foundation stone of cellular irritability." His conception bears close inspection because it says that disease strikes at one of the very fundamentals of life, irritability. Disease, as we have said, is a process. A pathogen is the irritant that keeps it going, that stokes the fires.

Parasite, the second half of the pathogen-parasite couplet will be discussed later under the coexistence problem of pathogen and host. Suffice it to say here that pathogen and parasite cannot be equated as they often are.

We are unable to trace very clearly the history of the term pathogen in plant pathology. We cannot find it in Ward's masterful treatment of plant pathology published in 1901. It is not mentioned in Duggar's book in 1909, nor in a 3-paper symposium in Volume I of "*Phytopathology*" published in 1911.

The first use of the term we can find is in Whetzel's book on the "History of Plant Pathology" published in 1918. The word did not appear in *Phytopathology* until 1921. In his Cornell lectures for 1925, Whetzel says that the word was of recent introduction and was derived from human pathology. It seems probable that Whetzel, with his flair for new words, introduced it some time between 1911 and 1915, probably in his lecture course. His student, Grossenbacher (1915), used pathogenic but not pathogen in a paper in 1915.

The term was spelled pathogene at first. Whetzel so spelled it, *Phytopathology* so indexed it, and Heald so spelled it in his classic text of 1933.

Phytopathology dropped the final *e* in 1932 and has spelled it *pathogen* ever since.

III. What Is Pathogenesis?

Because a pathogen is an agency that generates suffering in the plant, pathogenesis is the process of generating suffering. Pathogenesis is the chain of events in the casualty of disease. It includes the action of the pathogen, the susceptibility of the host, and the impact of ancillary factors.

That quality, that property, that ability of a pathogen to generate disease is pathogenicity. This is an abstract term that carries no implication of how disease is caused. We are unable to find any substantive difference between pathogenism and pathogenicity and we conclude that they are synonyms.

IV. The Kinds Of Pathogens

The agencies that generate suffering are numerous and they are diverse. One is tempted to say with Stakman and Harrar (1957) that pathogens are animate and inanimate. This is a simple understandable distinction but it leaves the viruses hanging. Viruses, as usual, lie in the twilight zone between. In any pair of categories, we have the yeses, the noes, and the maybes. The viruses are the maybes. Therefore, suppose we give them a class of their own and say animate pathogens, inanimate pathogens, and viruses.

According to Whetzel's lecture notes, the word pathogen was borrowed from our medical colleagues to signify animate pathogens, especially lower order organisms like fungi and bacteria. This idea was a worthy one and it filled an important need in the language of the mycologists and other microbiologists during their heyday at the forefront of the science of plant pathology.

The word pathogen is a more comprehensive term than that, however, and its very origin enables it to cover a wider range of things than microorganisms. If a pathogen is an agent that generates suffering, we are mentally myopic if we continue to limit it to microbial agents that generate disease. We hope that the word will expand to the limit of its denotation—that it will be a generic term to cover all agencies that generate disease by continuous irritation.

A. *Animate Pathogens*

The animate pathogens need little discussion. They have been at the forefront of plant pathology for scores of years. The animate pathogens are usually microbial, but many diseases are caused by small animals such as nematodes, arachnids, and insects.

An insect is an animate pathogen and, on that account, insects have been recognized the longest. Theophrastus (see Whetzel, 1918) wrote about the insects that cause plant disease. Insect pathogens to this day are generally included in plant pathology in Europe but seldom or never in the United States.

This is one of those unfortunate cases of bad leadership at the time when plant pathology departments were set up. Farlow introduced plant pathology to America about 1875, and first taught it. He was a mycologist and emphasized mycology. He left the insects and arachnids to the entomologists. They have handled the subject reasonably well, but they are zoologists first and plant pathologists last. The pathological processes that insects and arachnids bring about are treated only occasionally by the entomologists.

Even to this day the insect-induced diseases seem to fall between the two stools of entomology and mycology. We believe that research on the water deficiency brought on by red spiders would be illuminating. Entomologists are unlikely to study it. This would repay study especially if made in conjunction with the effects of powdery mildew fungi on the water economy of tissues.

The galls caused by insects could stand a vast amount of additional research especially in relation to crown gall, cedar gall, and the like. Aphids and leaf hoppers produce serious leaf diseases with attendant effects on starvation of the tissues. The field is wide and almost untouched.

In thus drawing attention to insects as pathogens, we do not wish to imply, however, that all insects that feed on plants necessarily produce disease. The Japanese beetle very badly injures a rose blossom and the Mexican bean beetle injures the bean leaf. These are injuries by our definition in Volume I, because the causal factor tends to be transient and the host quickly recovers as from other wounds.

The nematode as a pathogen is philosophically fascinating. In general, the mycologically minded plant pathologists refused at first to include such small animals as nematodes within their purview. After all they are not fungi. Similarly, the entomologists eschewed them also. Eventually, as mycology lost some of its grip on plant pathology, the nematodes came to be considered as the responsibility of plant pathologists. A few universities by now have set up departments of nematology. Whether this is a sound move or not seems uncertain as yet.

The suffering to the plant that nematodes cause is likely to be neglected in nematology departments if for no other reason than that a poker hand is what you call it. If the department is labeled nematology, it will study round worms and will deemphasize the diseased host. The members of the department are likely to neglect the pathology, in the same way that mycologists, bacteriologists, and entomologists have.

The microbial pathogens play the leading role in pathogenesis. Fungi probably produce more plant diseases than all the rest together. Bacteria also cause many plant diseases.

B. *Virus Pathogens*

The virus pathogens were investigated first by non-mycological plant pathologists. By now they have become so scientifically fascinating and so practically important that they are finding their own place in the sun. Journals of virology have been introduced. Eventually, we may come to have university departments of virology.

C. *Inanimate Pathogens*

The idea of the inanimate pathogen has slowly gained momentum in plant pathology. It seems to have reached print in the book of Stakman and Harrar (1957). The idea seems worth exploring here.

Very soon after the germ theory of plant disease was settled for plant pathology by De Bary (1853), exceptions began to appear. Diseases were discovered for which no living germ could be found. More of these diseases have been described. Some were known long before the germ theory. In human medicine we have for example, scurvy, diabetes, and pellagra. In plant pathology we have whiptail of cauliflower and apple scald as examples.

These diseases have their causes just as surely as fire blight on apple or late blight on potato. The difference is that the causes are inanimate, not animate. Whiptail is now known to be due to a deficiency of molybdenum in the soil, and apple scald is due to the gaseous products of respiration of the apple in cold storage.

We believe that the word pathogen should be extended to cover these inanimate causes of disease. There may be those who will argue that a deficiency of molybdenum is not a pathogen. This is equivalent to saying that zero has no reality. Without it, mathematics would still be in the Dark Ages.

V. PROVING PATHOGENICITY

To prove the pathogenicity of a given irritant may be exceedingly difficult. Generally speaking, a disease is identified by its own special symptoms and these are reasonably constant in type and magnitude. This is called the symptom picture or syndrome. The diagnostician infers the nature of the disease from the nature of the symptoms and guesses what pathogen is involved. If he suspects that a parasite is the pathogen, he examines the tissue macroscopically or microscopically for bacteria, fungus spores, fruiting bodies, mycelia, insects, arachnids, and nematodes. If he suspects that the pathogen is a mineral deficiency or excess, he runs leaf or soil analyses for the suspected element.

If the suspected pathogen is invariably associated with the disease or with the symptom picture, he is reasonably sure he is on the track. If possible, he cultures the suspected organism, notes its characteristics in pure culture, and inoculates it onto healthy hosts. If the original syndrome or symptom picture is reproduced, he is even better assured of the accuracy of his diagnosis. Finally, he recultures the organism. If the reculture is identical with the first, the diagnostician says he has fulfilled Koch's rules of proof.

Many pathogens, both animate and inanimate, cannot be subjected to all of Koch's rules. Many living pathogens are difficult or impossible to put into pure culture. These include the rusts, many smuts, mildews, nematodes, even insects. Here one must skip the culturing part of the proof. One satisfies himself by transferring the organism from a diseased to a healthy host. He proves that the "disease is catching" by proving that the organism can move from host to host. Very often a reversal of this process is helpful in proof. Since a disease keeps going only by continuous irritation, removal of the irritant should arrest the disease. If so, this should be as good a proof of pathogenicity as applying the pathogen in the first place. As Allen shows in Chapter 15 of this volume, the pathogenicity of many nematodes has been proved by this device.

Similarly, diseases due to mineral deficiencies and chronic diseases due to excesses of such substances as SO_2 and fluorides can be investigated and pathogenicity proved by adding or subtracting the suspected agent.

One is impressed with the long history of the alleged pathogenicity of so-called toxins in plant diseases as discussed by Ludwig in Chapter 9 of this volume. Dimond and Waggoner (1953) pointed out that few, if any, investigators have attempted to apply Koch's rules of proof to toxins. Woolley *et al.* (1952) have done this with the toxin for the wildfire disease of tobacco. The proof is (a) constant association, (b) isolation and identification of the toxin from diseased lesions, (c) production of the toxin in culture, (d) production of typical symptoms on the pure compound, and (e) reduction of symptoms by therapy with a toxin antidote. One must take a dubious view of the pathogenicity of all alleged toxins until all or most of these steps have been taken.

A. *Impact of the Germ Theory on Disease Names*

When De Bary and Pasteur succeeded in showing that diseases are caused by pathogens, they opened a Pandora's box of unprecise and ungrammatical names. Their use contributes its bit to the dictum that science is untranslatable.

Many diseases were named so long ago, so long before De Bary, that the origin of the names disappears into the limbo of the earliest languages. Blight is so old that the lexicographers throw up their hands. Blast, bunt, gall, rot, rust, and wilt can be traced to the old Saxon or Scandinavian roots. Scab derives from the Latin, to *scratch.*

Diseases are named also by their symptoms. From symptoms we get such disease names as fire, scald, scorch, witches'-broom, spot, and yellows.

Sometimes basic words are qualified to describe symptoms and we get names such as fire blight. We have deep scab, shallow scab, scurfy scab, and powdery scab. We also have red rust, black rust, orange rust, and yellow rust.

We often call on the host in naming diseases. The host can be combined with one of the ancient old names, to give a name like tomato wilt, with a symptom to give cabbage yellows, or with both to give fire blight of apple. We obtain still more names by using a part of the host to give us names such as root rot, leaf spot, or twig blight.

The combinations and permutations of all of these provide a fantastically large assortment of names but not enough. The advent of the germ theory of disease explosively expanded the need for new names. Most of the old words turned out to be too general. The wilt disease of tomato proved to be a conglomeration of at least four diseases. We in effect now had to name an enormous number of host-pathogen combinations. This was forced upon us. We had to name them or we would flounder in confusion. Curiously, in our efforts to avoid confusion, we have tended to add to it.

We borrowed one useful device from our medical colleagues. We named some of the new diseases in honor of the discoverers. This gave us Stewart's wilt of corn, for example. This was a good practice. It brought no confusion in its wake. And besides, it was good advertising!

The practice, however, never really took hold and has been abandoned. Instead, we fell into the easy, dangerous, and confusing habit of naming the new disease for the pathogen that causes it.

At first this seemed so reasonable and workable under the principle that each disease has a specific casual agent. It seemed so simple in the mid-nineteenth century for a mycologist to refer to the *Phytophthora* disease of potato, and to diseases collectively as fungus diseases. Like Humpty Dumpty, he knew what he meant even if he did do violence to the language.

He did violence to the language because up to that time animal disease, tomato disease, silkworm disease meant "a disease of animals, tomatoes, or silkworm." *Phytophthora* disease really meant then disease of *Phytophthora*. Darpoux discusses true fungus diseases in Chapter 13 of Volume III.

Unwittingly, the mycologist had begun to paint his floor from the door toward the back. He painted himself still farther into the corner by saying potato blight (*Phytophthora infestans*). When he got this far, he was equating disease and pathogen with all of its attendant dangers as already discussed in the Prologue of Volume I.

The confusion in this type of naming reaches a high point in the four wilt syndromes on tomato. The four pathogens are *Fusarium oxysporium* f. *lycopersici*, *Verticillium albo-atrum*, *Pseudomonas solanacearum*, and *Juglans nigrum*. We call the first and second *Fusarium* and *Verticillium*, wilt, respectively. The third we call bacterial wilt, and the last we try to sweep under the rug. If we say walnut wilt or *Juglans* wilt, we know that people will conclude that we are speaking of a disease of the black walnut. We have to say tomato wilt caused by the walnut.

Duggar (1909) must have sensed that mycologists were painting themselves into a corner, because he tried to bridge his way out by using the adjectival form. He titled his book "*Fungous diseases*" of plants, not fungus diseases. The suffix *-ous* means possessed of. A petalous plant is one that has petals. A disease is possessed of no fungus. A disease is a process.

Others, on occasion, have attempted to bridge their way back to the door over the fresh paint with another adjective, fungoid. The suffix *-oid* means to resemble. No disease resembles a fungus and, hence, cannot be fungoid.

Perhaps, the best compromise is to use the suffix *-al*. This means characterized by. The latter usage has reached complete adoption for bacterial diseases and to some extent for viral diseases.

It seems fair to say that late blight of potato is characterized by *Phytophthora infestans*. This does not equate the pathogen with the fungus. It does not say that the disease has the nature of a fungus or resembles the fungus, only that it is characterized by it. Koch's postulates say that the pathogen must be constantly associated with the disease. If so, then the disease is characterized by the pathogen.

In that case we would have the following names: fungal diseases of plants, fusarial diseases, phytophthoral diseases, bacterial diseases, and viral diseases.

B. *Naming Diseases Caused by Inanimate Pathogens*

Inanimate pathogens are very much more common than mycologists ever suspected. What to name them? This problem has bedeviled plant pathologists for a century. It has all of the same built-in problems as discussed above for naming diseases caused by living pathogens and it has other problems besides.

The commonest name for this great group of diseases is "physiological disease." This does make use of the *-al* suffix and it carries the connotation that the disease is characterized by physiology, presumably by abnormal physiology.

This term leaves something to be desired. Melchers (1915) clearly saw that physiological disease is a misnomer, because he said that "in truth all plant diseases are physiological phenomena" as Ward (1901) had so well said several years earlier. Melchers advocated the use of the term nonparasitic disease. The trouble with this term is that disease is a process, and a process can be neither parasitic nor nonparasitic. Melchers could have said nonparasitic pathogen. This is equivalent to our term inanimate pathogen.

We are unable to find a term that exactly covers the need. Noxal could apply to the diseases caused by poisons as for example the toxin from the walnut or the fumes from a smelter. Noxal could not apply to cracked stem of celery.

Lacking a really suitable word, physiological disease will have to suffice until a better one is coined. From an etymological point of view, physiologic disease would be preferable. Whetzel, in his lectures, accepted the term and used physiogen to label the pathogen.

VI. The Coexistence Problem in Disease

Because most diseases derive from animate pathogens, one of the big problems of plant pathology is to understand the nature of the coexistence of the two organisms, host and pathogen. This is discussed by McNew in Chapter 2 of this volume.

Coexistence for other organisms seems to have essentially all the basic problems of coexistence for people. At least the problems are closely analogous. Living together inevitably involves a certain amount of conflict which varies from the benign to the severe.

A. *Food Procurement Problems of the Pathogen*

The animate pathogen has problems of food procurement. Most animate pathogens have no chlorophyll, and hence, they must procure previously elaborated food. They may feed on dead organic matter and kill the host to get it, or they may be so finely adapted to their hosts, that the host hardly knows it is producing food for two.

Parasitism is a word bandied about in plant pathology. It becomes a very useful word when it is used in the context of food procurement. Parasitism is the ability of the one organism to procure food from another.

If the host is damaged, thereby, then we say that the organism has a faculty for pathogenicity. It can generate disease.

B. *Grades of Commensalism*

The coexistence of two organisms is commensalism. This means dining together at the same table. The word comes from the Latin words *com*, together, and *mensa*, table.

If the commensalism is cooperative and both contribute to the food supply, we have symbiosis. If one partner supplies all the food and the other contributes nothing, we have parasitism. If one produces damage while it feeds, we have pathogenism.

At some admitted risk, the editors suggest the analogy of the mother-in-law who lives commensally with you. If she is invariably a nice, pleasant old lady who pays all her share and never complains about how you raise your children, you have symbiosis. If she is never in the way, but must depend upon you for food and shelter, you have parasitism. If she is constantly raising hob in the family, you have pathogenism whether she pays her way or not. If she does pay her way, she is a pathogen but no parasite. If she does not pay her way, she is both a parasite and a pathogen.

In the plant kingdom, we can find examples of all grades. The fungus and the alga of a lichen coexist symbiotically. The *Lolium* fungus and the so-called virus of healthy potatoes coexist with their host parasitically, not pathogenically. A vine or Spanish moss coexist with a tree as pathogens but not as parasites. Root rot fungi are very close to this situation. They coexist generally as pathogens and not as parasites. They must kill the host before they can feed on it. Probably most organisms coexist with their hosts as both parasites and pathogens.

VII. THE LONG STRUGGLE TO ESTABLISH CAUSALITY

The story of the long, long struggle to establish the role of pathogens in the causality of disease is ever fascinating. It is the story of the slow clearing of some very muddy water.

The abstract concept of disease was not difficult to derive and ancient men must have arrived at the distinction between disease and injury. An appreciation of cause and effect is also ancient. The tormenters of Socrates knew quite well that his death would follow the drinking of the hemlock tea.

The struggle to establish causality for diseases dragged on over many centuries for several reasons. (1) It was difficult to distinguish the real cause for disease from the action of ancillary factors that also are involved. (2) The sequence of events was confusing. This, to a considerable degree, is the hen and the egg dilemma. Which comes first, the hen or the egg? A very basic problem was that the participants did not, probably could not, even know that they were confronted with the hen and the egg dilemma. (3) This leads us to the third difficulty. Most of the eggs in this dilemma were too small to be seen. That is to say the fungi, the bacteria, the viruses, the minerals were too small to be sensed visually for many centuries.

A. *Action of Ancillary Factors*

The ancients, of course, searched for causes among the things current in their environment. The ancient Hebrews and the Romans believed that an angry God visited diseases on a farmer's crops as punishment for his sins.

Waggoner in Chapter 8 of Volume III points out that diseases are varied. He says that weather is as common as, and more variable than sin and, hence, weather, especially bad weather, must be involved in plant disease. Theophrastus, 23 centuries ago (see Whetzel, 1918), did not miss this obvious correlation. Said old Theophrastus, "lands which are exposed to the wind and elevated are not liable to rust, or less so, while those that lie low and are not exposed to the wind are more so." In other words rust occurs in a "foggy bottom."

Now this was quite obvious also to the Irish in the wet, wet weather of 1845, 1846, and 1847. The blight disease on the potato was terrible and it, too, started first and was worst in the foggy bottoms. The land was wet, soggy, and oozy, and the potato leaves were wet, soggy, and oozy, too. *Ergo,* the weather must have produced the disease.

The trouble with this argument, of course, is that disease is more variable than either weather or sin. The causes must be as variable as as the diseases. Otherwise, one runs out of causes before he runs out of diseases.

Another difficulty, of course, is that weather is variable enough to account for a few of the numerous diseases. When the Bible scribe wrote that the East wind blasts the grain, he really meant just that. This was the action of drought as a continuous irritant. It was exaggerated by the excessive drying of the east wind off the desert.

Similarly, "wet feet" causes disease in plants, and so does excess acidity or alkalinity in the soil. An excellent example of the latter is the head rot disease of cauliflower that occurred in the Catskill Mountains in the mid 'thirties. An excessively high concentration of hydroxyl ions in the soil immobilized the boron. Without boron, the phloem in the cauliflower plant was blackened and the head autolyzed into a slimy mass.

B. *The Problem of Sequence*

The cauliflower disease exemplifies the problem of sequence in the causality of disease. If one gets his "cart before his horse," he cannot move.

Perhaps the first "break" in the long history of the search for causality came with the suggestions that poisons are the continuous irritants that are responsible for plant disease.

Poisons are often volatile. We have known about the oil of bitter almonds for centuries. Timothy Dwight, amateur plant pathologist, and erstwhile president of Yale University settled the wheat rust problem in 1796 (see Hedrick, 1933). Said he, " . . . [the barberry] is generally believed to blast [grain]. Its blossoms emit very copiously, a pungent effluvium, believed to be so acrimonious, as to injure . . . both kinds of grain." This accounts for the barberry law in Connecticut in 1726. This is the grandfather of all laws in America on the public control of pests, human, animal, and plant. "If thine eye offend thee, pluck it out." And the barberry was offensive. Therefore, pluck it out.

In 1752, Tillet (1755) actually saw the spores of the wheat smut fungus and he actually inoculated the seed and experimentally produced a smutted crop. He was so imbued with the poison theory of causality, however, that he considered that the smut spores are but the carriers of the poison.

And similarly Morren (1844, see Johnson, 1935), the Belgian, was sure that potato blight results from the putrid smelling miasma (poison) arising from the dank soil in the diseased potato fields.

By the middle of the last century, Darling, the mayor of New Haven, Connecticut, a lawyer, came closer than any of his fellows to deducing the abstract concept of causality. Of course, being a lawyer he was, perhaps, better versed in the rules of evidence than some of his biological confreres.

His Honor, the Mayor (1845) was concerned about peach yellows in his garden. He was impelled to the conclusion that it is caused by a poison. He was 50 years or more ahead of the biologists who deduced about 1900 that such diseases as peach yellows are caused by "filterable enzymes" subsequently called viruses. The pristine definition of virus is "poison." In effect, Darling said that peach yellows is caused by a virus.

If Erwin F. Smith had read Darling's paper, he could have saved himself many years of research on peach yellows. Darling said "whence comes this poison, if it be such? I will hazard a conjecture that it is derived from some unknown insect." Shades of Theobald Smith and M. B. Waite!!

Thus, the poison theory led on into viruses. It hindered somewhat the discovery of the causal role of microbes, however.

Of course, the really critical discovery in the long, long road to a deep understanding of the nature of causality was the discovery of microbes by Leeuwenhoek in 1683. This invention guaranteed the solution of the major enigma of disease causation, the role of microbes. It was now only a matter of time.

In 1725, 40 years after Leeuwenhoek, Professor Bradley of Cambridge University examined a smut ball from wheat. He saw minute black bodies. He inferred correctly that they were causal, but he considered that they were insect eggs.

In 1807, Prévost used the same experimental designs and the same spores as Tillet but Prévost correctly inferred that the smut spores were causal. When he inoculated them to wheat seed, he recovered their progeny in the grains of the wheat that grew from the inoculated seed.

To many biologists, Prévost had made a preposterous claim and it had to be subjected to "the team approach." It had to be put on trial before a committee of the august French Academy of Sciences. "Impossible," they said.

The trouble with the learned men was that they believed that the smut spores were excrescences from diseased tissues. Had not the great Zallinger (1773) said this so long ago as to give it the patina and authenticity of age? These things come after the disease, do they not? *Ergo*, they are caused by the disease. The academicians got the cart before the horse.

Anton De Bary (1853) was not impressed with the committee action, not impressed with majority rule. He proceeded to inoculate wheat seed with smut fungus. He followed the mycelium up the plant and into the new seeds. Thus, he unhooked the horse from behind the cart and put it in front. He placed in proper order the sequence of events of causality. Microorganisms do, indeed, cause disease. They are not like toadstools that arise from decaying vegetation. They are toadstools that cause the decay.

This returns us to the hen and the egg dilemma. The fungus causes the disease, but on the other hand the disease precedes the fungus, too. If you are a broiler producer, you believe that the egg comes before the chicken. If you produce eggs for the grocer, you believe that the chicken comes before the egg.

The pathologist studies disease. To him the disease follows the pathogen and is caused by it. This view enables him to control disease. He has controlled more disease in the hundred odd years of the germ theory than in the million odd years of the sin, weather, and poison theories.

Thus, the chain of casuality was finally established, → fungus → disease → fungus → disease.

We then knew why disease was said to be "catching." It is the fungus that is "catching." The fungus is what moves. Everything now fell into pattern, the barberry enigma for wheat rust, the wet miasma for potato blight.

The establishment of bacteria as casual agents followed shortly on the heels of the discovery of the oil immersion lens for the microscope in 1844. We now had three points in a size scale of organisms that cause disease: insects, fungi, bacteria. There must be still smaller ones. These were soon discovered and called viruses. They were not actually seen until some 40 years after they were postulated, however. Their visual observation had to await an advance in physical technology, the electron microscope.

All of this was very satisfying. It removed the mysticism from disease. It simplified the conceptual schemes. It gave the practical man a procedure for practical control. He had to aim his guns at the casual agent, at the pathogen.

Moreover, in plant pathology it gave the mycologists a perch from which they have directed the destinies of plant pathology for nigh onto 100 years. An astonishing number of authors in this treatise, more or less unconsciously, used in their first drafts the word parasite to mean pathogen. This has two causes: (a) it is a carryover from the mycological era, and (b) the vast majority of plant parasitic microbes are pathogens.

C. *The Recurring Conflict with the Weather*

By 1915, the animate pathogens had been pretty well described and ambitious plant pathologists began to look for progress elsewhere. One of these was L. R. Jones. Jones spent his scientific manhood in the rigorous climate of Vermont where the weather is highly important. The weather can freeze a man to death in the winter. If the frost comes too late in the spring or too early in the autumn, a farmer could be ruined.

When Jones went west to Wisconsin "to grow up with the country" he decided that he must investigate the effect of weather on plant disease.

Shortly after he established the department at Wisconsin, he was elected president of the Botanical Society of America and he wrote

(Jones, 1914) "The relation of environment to the predisposition of the host, as to the virulence of the parasite cannot be overemphasized." It has been the trademark of Wisconsin plant pathology, the subject of innumerable doctoral theses.

Such a volume of work inevitably leads to changes in philosophy, perhaps to changes in the names for things. The Wisconsin weather work has led to the rise of a new word: a pathogen no longer causes a disease, it *incites* it.

We and many other plant pathologists agree with Luttrell (1954) that the verb to incite is unnecessary—that the old fashioned word, cause, is sufficient.

Cause is a larger word than incite. The synonyms for cause are originate, occasion, give rise to, produce, bring to pass, create.

Incite derives from the Latin root, *cieo,* to call. It means to call out, to investigate, stimulate, arouse, urge, step up, goad, inflame, kindle, or foment. This is clearly a subdivision of cause. To incite is to cause, but to cause is not to incite. Unfortunately, some pathologists use incite as a synonym of cause.

Webster's new international Dictionary discusses "cause" as follows. "When we scientifically state causes we are describing successive stages of a routine of experience; causation, says John Stuart Mill, is uniform antecedence, and this definition is perfectly in accord with the scientific concept."

If plant pathology is a science, then diseases are *caused* and not fomented, encouraged, aroused, goaded, or *incited.*

The sense of incite is to get something started. That is probably why it is most commonly used in the sense of "to incite to riot." One incites a mob to riot. A riot is something that feeds on itself. In fact, the inciter may even try to stop a riot that he has incited. He usually fails simply because a riot is self-perpetuating. The inciter of a riot can even be killed in the incitement without affecting the course of the riot.

Diseases are not self-perpetuating. Only the pathogen is. On that account diseases cannot be triggered, kindled, or incited. In fact, if the pathogen that causes a disease is removed, the disease stops progressing.

The only disease we know that is incited is crown gall. Once the gall is going, the bacteria can be removed by heat. The gall goes on just the same. It has been incited.

Weather is indeed a factor in pathogenesis. Late blight of potato is very closely geared to the weather. The night of infection must be cool and humid. The day following must be warm. Unless the weather is favorable, one can inoculate potatoes every night with sporangia of

Phytophthora infestans and no disease will result. Once inoculation is successful and infection begins, however, weather plays no significant role as long as it remains within the growth range of the potato.

In this case, then, weather, not the fungus, incites the disease. Remove the special weather; the disease continues. Remove the pathogen; it stops.

The pathogen is the basic cause. It must be there. As we have said, the pathogen characterizes the disease. The weather does not. The pathogens are as numerous as the diseases. The weather variations are not.

Occasionally, of course, as in drought, weather can also be the pathogen and in that case weather is the cause of disease. Usually, it is not.

VIII. The Organization of Volume II

Having discussed the basic design of Volume II, having attempted to put the pathogen in its proper perspective, perhaps a few words are in order on the details.

The volume is really divided into two basic sections, parasitism and pathogenism as defined earlier. We have assigned only one chapter to parasitism. It is devoted to the nature, origin, and evolution of parasitism. McNew treats it from the point of view that increasing refinement of parasitism is essentially matched by a concomitant reduction in pathogenism. A highly pathogenic organism like *Pythium ultimum* or *Erwinia cartovora* promptly slays its host and lives on the carcass. This is not a very high level of parasitism. The rusts and smuts, however, have become very sophisticated parasites. They keep their hosts alive as long as possible. They do not "kill the goose that lays the golden egg."

The rest of the volume deals with pathogenicity, the ability of the parasite to produce diseases. Seldom is the word parasite used, however, in the text, because we are writing a treatise on plant pathology not on parasitism. Our treatise deals with the generation of disease in plants, not with the physiology of the food gathering process by a parasite.

We deal with the pathogen in three sections: reproduction, the nature of pathogenicity, and the mechanisms of inhibiting the pathogen.

The section on reproduction contains chapters on the prime pathogens, viruses, bacteria, fungi. It also contains a chapter on spore germination, primarily because of the richness of data in this field and its importance to practical plant pathology. No chapter deals directly with reproduction in nematodes because the information is meager as yet. Allen covers this subject in Chapter 15. Similarly, there is no chapter on reproduction in insects and arachnids. This is covered in texts on entomology.

We have devoted six chapters to pathogenicity. These are six chapters on the ability of the organism to cause disease. Five of these are straightforward. The first two, mechanical ability to breach host barriers and chemical ability to breach host barriers, are the opposite numbers from the two in Volume I on mechanical and chemical defenses of the host. The similarities and contrasts here are interesting.

The pathogenicity of toxins is discussed in Chapter 9, strains of the pathogen in Chapter 10, and genetics of pathogenicity in Chapter 11.

Only Chapter 8 fails to fit smoothly into this scheme. It deals with soil organisms, the roots, and the interaction in the rhizosphere. At first we had no such chapter, but several advisors recommended it because it is a fascinating field and the phenomena are slightly different from those for aboveground diseases.

It does, however, inevitably involve some overlap. We hope that the stimulating presentation will compensate for this.

The four chapters on inhibiting the pathogen are straightforward. They concern themselves with inhibition of viruses, fungi, and nematodes. Again insects and arachnids are neglected for the sake of convenience. Fungicides are treated in two chapters—one from the point of view of the chemist, the other from the point of view of the fungus.

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CHAPTER 2

The Nature, Origin, and Evolution of Parasitism

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Any interference with the normal metabolism of a plant that prevents it from making full use of the factors available in its environment for growth and reproduction must be considered as a disease. The agent that interferes with the physiological processes of a plant is a pathogen regardless of whether it is living or inanimate.

The living pathogens have been of much more concern to plant pathology than the inanimate ones because of their ability to multiply and create epidemics. Most of the living pathogens are parasitic because they invade one or more plant tissues to procure food but parasitism is not a prerequisite to pathogenicity. As a matter of fact the processes of parasitism and pathogenicity in an organism often are entirely different and specialization for one attribute is often made at the expense of the other.

I. INTERRELATIONSHIP OF PARASITISM AND PATHOGENICITY

The interrelationship of parasitism and pathogenicity can best be interpreted by considering the pathogen and the injured plant in a disease association as symbionts. In the classical sense employed by De Bary (1879), there is symbiosis whenever two organisms occupy the same habitat. In a few unique associations the symbionts are mutually independent and nonresponsive to each other but usually they affect each other's development. The physical contact of symbionts ranges from disjunctive to fully conjunctive and their effects upon each other vary from mutual or unilateral antagonism to synergism. The following illustrations of unilateral, antagonistic symbiosis will serve to define the range of pathogenic processes.

Very few, if any, organisms can occupy the same habitat as another without influencing its growth. For example, the sugar beet inhibits the germination of seed of peppergrass in its immediate vicinity presumably because of the toxic materials it secretes such as *p*-hydroxybenzoic acid, vanillic acid, ferulic acid, and *p*-coumaric acid (Massart, 1957).

The soil sickness resulting from the growth of wheat, rye, and other crops is caused by picolinic acid and other chemicals in the soil (Schreiner and Sullivan, 1909). The glycosides and phenolic substances from an apple or peach root (Patrick, 1955) prevent replanting of younger trees in an old orchard. Juglone and its trihydroxy naphthalene analog from roots of the black walnut tree poisons the soil for many other forms of plant life such as tomato and cabbage which become chlorotic and die (Massey, 1925). Such plants cause disease in other plants and, hence, must be considered as pathogens even though they do no more than foul the environment with by-products of their metabolism. This is pathogenesis by independent association of two subjects, but it is not parasitism.

Most pathogens, however, have a much more intimate relation with the infected plant based upon a nutritional affiliation in which one serves as the host to the parasitic endeavor of the other. A plant parasite is any form of animal or plant life that invades a plant and multiplies or grows at its host's expense without contributing much, if anything, in return. The arbitrary removal of water, mineral elements, or synthesized food from the metabolic pool of the host usually will prove detrimental to its further development, so parasitism in most plants automatically leads to pathogenesis.

For example, the mistletoe of ponderosa pine is a pathogen even though it is fully capable of synthesizing its own foodstuffs. It creates disease by interfering with movement of minerals and nutrients to the foliage and possibly by adding injurious materials to the tissues of the pine tree.

Many parasitic establishments do not become pathogenic if the parasite compensates for the damage done by its presence. The mycorrhizal fungus certainly is a parasite on the root of trees but it may be relatively noninjurious or even may be beneficial in some soils by increasing the absorbing area of the root so as to expedite uptake of mineral nutrients from the soil (Burges, 1936; Hatch, 1937; Funke, 1942). By strict definition this is a commensal association rather than parasitism since the invader contributes to the welfare of its host.

There may be other auxiliary benefits from parasitism that completely compensate for the damage done to the host. The legume nodule bacterium (*Rhizobium leguminosarum*) causes a disease reaction in the nature of a proliferating gall which undoubtedly diverts foodstuffs from normal cell metabolism and orderly growth. However, the extra atmospheric nitrogen fixed by the nominally parasitic bacteria eventually proves to be so beneficial to host development that the relationship must be considered to be a mutually beneficial symbiosis or commensalism of bacterium and higher plant.

It follows that the symbiosis between the bacterium and its host is more likely to be beneficial to a legume growing in a nitrogen-deficient soil than in one supplied with adequate amounts of nitrogen. As a matter of fact, if soil nitrogen were present in optimum supply for growth of the legume throughout the season, it is conceivable that the nodule bacteria might be considered as a gall forming, pathogenic parasite rather than as a commensal symbiont.

These brief examples illustrate the basic facts regarding pathogenesis. Plant diseases so caused are the product of an interplay between the metabolism of the plant, physiology of the parasite or other symbiont, and the environment. In a few exceptional associations, there can be parasitism without severe pathogenesis, and disease induction without parasitism. Parasitism usually is an attack on organic food reserves by a chlorophyll-deficient form of plant life but it certainly is not restricted to these primitive forms of life or this one class of nutrients. The intensity of the disease reaction will be determined by the physiological balance existing between the two symbionts as mediated by the environmental forces operating upon their symbiosis.

II. The Physiology of Pathogenicity

In one broad sense, plant pathology is an aspect of plant ecology. Like all other ecological sciences, it must turn to physiology and biochemistry for the ultimate answers as to why plant societies behave as they do. However, a plant disease is something more than the sum total of the physiology of the host and the pathogen. It is the physiology of a symbiotic state in which the biochemical activities of pathogen and host are completely consolidated.

A. *Appropriation of Nutrients by Pathogen*

There are considerable data on the physiology of the fungi (Foster, 1939, 1949; Wolf and Wolf, 1947) and bacteria (Dowson, 1957) that cause plant diseases. Unfortunately, however, there are all too few data on the biochemistry of their parasitic activities and pathogenic capabilities while in the host (Gäumann, 1950; Allen, 1954; Lilly and Barnett, 1951). The measure of their parasitic activity should be ability to remove different classes of foodstuffs from the host.

B. *Toxin Formation by Pathogen*

The loss of food materials undoubtedly constitutes a drain on the metabolic activities of the host and leads to inefficiency in its growth and reproduction. However, most parasitized plants are damaged much more than would be expected from the drain on their metabolism in supplying nourishment to the invader. This comes from increased respiratory activity of the invaded tissues, disintegration or collapse of the adjacent tissues, and various physiological stimuli that cause wilting, cell proliferation, cell elongation, abscission of leaves, and degeneration of chlorophyll.

These effects are the results of the extracellular materials secreted by the pathogen or incited to production by the infected cell. Gäumann (1954) refers to these materials as toxins but they could be appropriately subdivided into more specific classes such as extracellular enzymes, auxins, wilt toxins, and cell stimulants. Those materials that regulate disease processes are attracting more attention but there is not enough known about them to permit the classification of disease according to the physiology of the pathogens.

C. *The Six Major Processes Affected*

Attention may be directed more profitably to the activities of the host as a basis of understanding disease processes because the basic knowledge on the physiology of higher plants is fairly well understood. It revolves around the synthesis, transport, use and/or storage of food materials, and the auxiliary services thereto. In its simplest terms, the chronological sequence of events in the six major processes is as follows: (1) A food reserve consisting primarily of energy sources in carbohydrates or lipids and secondarily in nitrogenous materials is used to nourish the embryonic tissues of seed, tuber, root, or bud after the dormant period. (2) Juvenile tissues that are dependent upon these reserves develop as seedlings or shoots. (3) Roots are established to procure water and mineral elements essential to growth, photosynthesis, and protein metabolism. (4) The nutrients and water supplies are transported to the leaves and growing points through the tracheal tubes. (5) The green leaves assimilate a new supply of carbohydrates by photosynthesis so the young plant becomes independent of the food reserve. (6) The products of photosynthesis are transported to the area of cell utilization where they are incorporated into new tissues or else are stored in seeds, buds, roots, or stems.

Thus, there are six vital steps in the life of the host, six vital processes. In this chapter we shall refer often to these six vital processes and the diseases thereof. Any one of the six may be injured or even wholly blocked by a pathogen.

If any one of the processes is brought to a stop, the plant succumbs but this is the exception rather than the rule. Ordinarily, the function is injured but not destroyed so only the efficiency of the plant is affected. A major objective of plant pathology is to learn how these processes are injured and to design methods of preventing or circumventing that injury. The science, therefore, has as its applied objective the conservation of foodstuffs and the promotion of maximum efficiency in their synthesis and utilization by the plant.

If the pathogens that disrupt each of these six processes are grouped together irrespective of their taxonomic derivation, a surprising degree of order is obtained. The ones in class 1 that do no more than attack and destroy food reserves are good pathogens but very poorly developed parasites. They are essentially opportunistic saprophytes. Likewise, the soil saprophytes that attack seedlings (class 2) and roots (class 3) are good pathogens and relatively poor parasites. They become harmful only when conditions handicap the host so as to keep it in a susceptible state. The vascular or wilt parasites in class 4 are facultative parasites with very definite parasitic specialization.

Some of the leaf invaders of class 5 in the Fungi Imperfecti are only slightly better parasites but a high state of parasitism is attained by some, such as the downy mildews. The powdery mildews and rusts are obligate parasites on foliage. They have lost all capability for an alternate existence as a saprophyte and have become so dependent upon the host's physiology that parasitic races are necessary for their survival. The pathogens in class 6 that have come to reside inside the host cells and regulate cell activities in the metabolism of proteins, cell division, and cell differentiation show an appreciable degree of commensalism regardless of whether they are obligate or facultative parasites.

Here, then, is a system for organizing the types of pathogenesis. It is based on disruption of physiological processes of the host. There is enough order and logic to this to warrant its further study. By so organizing the knowledge on plant diseases into an integrated system depending upon function, certain natural laws of pathogenesis can be evolved that are valuable in guiding research into fruitful channels and in organizing knowledge so it can be learned from principles rather than by rote.

The basic tenets of this system are that: disease is an abnormal physiological process in plants; the physiological processes are constantly in a state of flux; there is an orderly progression of parasitism depending upon which function of the host is under attack; and the system is natural enough to permit development of some general laws and principles for understanding disease and its control. Various aspects of these concepts are examined in the following sections.

III. The Natural Processes of Pathogenesis

A plant is diseased whenever its normal functions are impaired so it cannot operate at maximum efficiency under prevailing environmental conditions. Inefficiency (i.e., disease) comes from the loss of an organ, destruction of food reserves, impairment of essential functions such as photosynthesis and translocation of foodstuffs, or abnormal utilization that results in abnormal growth. For example, the abscission of a leaf attacked by a blight organism or the severance of roots by a rot organism destroys the normal balance between the water-supplying and food-synthesizing capacity of a plant. As soon as one is destroyed, the other cannot operate at maximum capacity so there is a loss of efficiency.

Disease is a physiological process regardless of whether a tissue is decayed or not. The blocking of photosynthesis, interference with translocation or induction of abnormal cell respiration is disease even though these abnormal processes may not be visible directly to the eye of the diagnostician.

A. *Interference with the Six Vital Processes*

The interference with the six vital processes of the host mentioned in the preceding section leads to six classes of pathogenesis. It is well to examine the nature of the pathogens and the six classes of disease they cause before proceeding to other considerations.

1. *Destruction of Food Reserves*

The pathogens that attack storage organs and organic material cause seed decay, timber rots, soft rot of vegetables, and fruit breakdown (see also Chapter 5 of Volume I). Most of the fungi and bacteria involved are very poor parasites and ordinarily live as saprophytes upon decaying organic matter. Most of them are habitual soil-inhabitors where organic matter provides carbohydrates and cellulosic materials but some are primarily aerial in habitat.

With very few exceptions their parasitic capabilities are limited to invasion of the storage organ through a wound or other unprotected tissue. Once inside the host tissue, they digest starches and cellulose by virtue of ability to secrete amylases and cellulase. In addition, many of them have ability to break down plant tissues by use of protopectinase and pectinase (Thornberry, 1938) as was first demonstrated for *Erwinia carotovora* by Jones (1909).

Among the typical examples of this class are the soft rots of vegetable caused by *Erwinia carotovora*, mold of drupe fruits caused by *Rhizopus nigricans*, soft rot of potato caused by *Erwinia atroseptica*, storage rot of lily bulbs caused by *Rhizopus necans* and dry rots of woody plants. In each of these, the host tissue is destroyed as a general cortical necrosis. As each cell succumbs it provides food to the parasite for further aggression. The host is essentially inert and does not respond to the aggression to any appreciable extent. These are potent pathogens but poorly developed parasites.

2. *Prevention of Seedling Metabolism*

The seedling blights are likewise caused by general soil saprophytes. Sometimes a parasite that attacks the seed will also invade the radicle or epicotyl through the wounds created by the emergence of adventitious or secondary roots. The pathogens on seedlings are one step more advanced parasites than the soft rot agents, since they do invade a rapidly growing, metabolically active tissue. As a rule, they are aggressive in juvenile tissue but may be restricted as soon as the adjacent tissue begins to achieve more mature differentiation.

Among the pathogens in this class are such common damping-off and seedling blight fungi as *Pythium ultimum, Pythium debaryanum, Rhizoctonia solani,* and *Diplodia zeae.* It should be noted that the latter two also can attack more mature plants. *D. zeae* is very aggressive on the seedling and often becomes established in the root crown of the corn plant where it remains relatively inactive until the plant is past its prime period of growth. The pathogen spreads aggressively again after the corn plant reaches maturity and begins to enter into senility.

3. *Interference with Procurement*

The root rotting diseases are caused by a wide variety of fungi and bacteria that are capable of either colonizing and enduring in soil or of invading it temporarily. They show some advance in parasitism over seedling pathogens since they can invade well-formed roots through lignified, mature tissue. Still, most of them are poorly specialized as plant parasites but they are quite aggressive pathogens. The routes of invasion are usually through wounds created by emergence of secondary roots, abrasion with soil particles, nematode penetration, or feeding of insects. However, some of them such as the cause of black root rot of tobacco, *Thielaviopsis basicola,* will form mycelial mats on the surface of the root and force a penetration directly through the epidermal wall by an infection peg that secretes enzymes or exerts direct pressure. Roots may be preconditioned to invasion by excess moisture or nutrient conditions such as are found in the brown root rot of wheat (Vanterpool, 1940). Apparently under anaerobic conditions during prolonged heavy rainfall, an accumulation of salicylic aldehyde predisposes the root to invasion by the fungus (Graham and Greenberg, 1939).

Many other factors in the rhizosphere influence the transition of the soil saprophyte into a pathogen. Apparently the organisms are stimulated to grow along the diffusion gradient of carbohydrates from roots. The mycelium grows more rapidly toward the source of food supply in the roots. However, the exudates from some roots, such as those from varieties of peas resistant to *Fusarium oxysporum* f. *pisi,* may be toxic to the pathogen and prevent its reaching the site of invasion (Buxton, 1957). Even the carbohydrates secreted by a root may encourage the growth of antagonistic soil saprophytes such as those that protect the cotton root from invasion by *Ozonium omnivorum* (Eaton *et al.,* 1947). The roots of cotton seedlings appear to be quite resistant to invasion because of the substantial amounts of carbohydrates in the roots, but quickly become susceptible as the sugar concentration declines during the period of rapid growth. It then seems to be resistant again in the advanced stages of its development (Blank, 1940) as sugar concentration increases sufficiently to affect the rhizosphere population.

In any evolutionary scheme, the root pathogens might be classified as the first strongly aggressive plant invaders. They include such destructive fungi as the species of *Pythium* on sugar cane, maize, and wheat; *Aphanomyces* spp. on sugar beets and peas; *Fusarium* spp. on pea, bean, and other crops; and *Ophiobolus graminis* on wheat. The ones of these that are natural soil inhabitants are difficult to avoid and are chronically destructive but the soil invaders that are poorly adapted to survival apart from their hosts in competition with other microorganisms (Reinking and Manns, 1933, 1934; Garrett, 1944, p. 13) may be avoided by proper treatment of the soil to favor their antagonists (King, 1940; Hildebrand and West, 1941).

The root rot organisms are poorly specialized parasites even though some have become adjusted to the rhizosphere environment and thrive best on or in roots. They are general cortical invaders with very little specialization in preference of tissue. The host has very poorly developed resistance. Most of the resistance exploited agronomically is in the nature of disease-escaping tactics where a particular variety has ability to develop new roots to replace those destroyed by the pathogen or has a favorable seasonal development that avoids the infection period.

4. *Interference with Upward Transport*

The vascular diseases or wilt diseases interfere with upward movement of water and mineral nutrients. They are caused by xylem parasites (see also Chapter 9 of Volume I). Some of them live in soil and have habits similar to those of the root rot fungi. They invade directly through root hairs or natural wounds, traverse the cortical tissues and become established in the tracheal tubes. Although they are only facultative parasites, they show a much higher order of specialization than the root rots in their preference of a specific tissue and for certain varieties of crops. They may also be considered more highly specialized because the typical disease symptoms are induced by response of the host to special causative chemicals.

Two classes of wilt organisms have to be considered, the soil-borne and the insect-borne. Many of them such as the *Fusarium* spp. on cabbage, cotton, tomato, watermelon, and banana are normal soil inhabitants. Others, such as the bacterial wilts of maize caused by *Bacterium stewartii* and of cucumber caused by *Erwinia tracheiphila* and the fungal wilts of oak caused by *Endoconidiophora fagacearum* and elm *Ceratostomella ulmi*, are insect-borne but they have essentially the same habits of invasion as the soil colonizers.

The masses of mycelium and bacterial cells present in the tracheal tubes interfere with the movement of water and nutrients and some tubes respond to invasion by forming tyloses that further complicate free movement of materials to the leaves. These mechanical impediments will not explain fully all the wilting symptoms so attention is focused upon the metabolic by-products of the parasite. There are a number of wilt-inducing toxins formed in cultures of a fungus such as *Fusarium lycopersici*, the cause of tomato wilt, which has been studied by Gäumann (1957); Davis and Dimond (1954), and Dimond and Waggoner (1953). The principal problem confronting these investigators has been to prove that enough of any one or any combination of these materials is present in the tracheal tubes at any one time to cause the wilt symptoms (see also Chapter 9 of Volume I).

The wilt organisms are fairly well specialized facultative parasites. There is a definite mechanism of pathogenesis above that induced in their normal parasitic activities. There are very specific forms of resistance in the host and it is possible to breed for such types of resistance. In any evolutionary scheme, these pathogens would rate rather high both as parasites and as pathogens with specific abilities; but there is no evidence that they and the host have any tendency to establish a commensal state. They cause general necrosis and frequently rupture the tracheal tubes and overrun nearby parenchyma cells.

5. *Destruction of Food Manufacture*

The leaf-invading pathogen (Group 3) include at least four diverse groups ranging from poorly specialized facultative saprophytes to obligate parasites. Most of the general leaf-blight organisms have ability to infect and destroy the stems, flowers, and fruits, as well as the leaves. Thus they can cause substantial loss in food manufacture by stem cankers, flower blasts, and fruit rots, scabs, anthracnose, etc.

This overlapping of parasitic activity on different organs is not surprising because the tissues involved are morphologically analogous. The flower, stem, and fruit may be considered as modified leaves that have assumed special functions other than photosynthesis. They have comparable epidermal cells and cuticle—at least while in the juvenile condition. The nectaries, lenticels, and hydathodes may be considered as having modified stomata.

The primary mechanism of pathogenesis is destruction of the food-synthesizing potential of the plant. This results from local necrosis of the leaf blade, which may become very extensive in some diseases such as late blight of potato, caused by *Phytophthora infestans*. In many other diseases, the damage is entirely out of proportion to the amount of necrosis because plants are defoliated by abscission-inducing substances such as those found in shot hole of peach caused by *Xanthomonas pruni* or are rendered chlorotic as in cherry leaf spot caused by *Coccomyces hiemalis*. Extensive rupture of the cuticle by the sporulating fungus may disturb the water economy of the plant with serious consequences such as often occur in wheat fields affected with *Puccinia graminis*.

a. *The Leaf Blights, Cankers, and Rots*. The leaf blights and stem cankers are caused by fungi and bacteria that range from very poorly adapted to highly specialized parasites but all are facultatively saprophytic. Taxonomically they are Schizomycetes, Fungi Imperfecti, and Ascomycetes. The lower members of the series are analogous to the root rot fungi in their parasitic potentials. As a matter of fact, some of them do attack roots and underground parts of the plants. The fire blight organism (*Erwinia amylovora*) may cause root cankers in the Pacific Northwest of the United States. Conversely, some of the root rot fungi may splash upon the stems of plants and cause stem cankers.

In spite of these overlapping tendencies it would seem well to separate the root rots and canker diseases because the mechanism of action is largely different, the habitat is usually entirely different, and there usually is more host specialization in the leaf invaders. There is no comparison, for example, in the parasitic specialization of *Pythium debaryanum* and *Venturia inaequalis*. In between these two are all degrees of specialization but usually there is one very significant difference. The root parasites have a well-developed mycelial stage whereas the leaf parasites have very little mycelial development apart from the host. The aerial parasite exists as a fungous spore or bacterial cell that penetrates directly or through a very restricted infection hypha. This becomes a major consideration in determining the nature of the parasitic establishment and the methods to be employed in controlling the two classes of disease agents.

b. *The Downy Mildews*. The downy mildews and their allies are caused by much better specialized parasites than the general leaf blights. The members of the Peronosporaceae, Albuginaceae, and Phytophthora are facultative to obligate parasites that establish liaison with the leaf tissue and maintain a more or less compatible relationship until the fungus begins to sporulate.

They usually have rather specific host requirements and there are sharp differences in the varietal reaction of plants to their invasion. It is possible to breed for disease resistance. The use of haustoria by the pathogen to procure food with a minimum of injury to the host appears for the first time in this group. In general, this group of pathogens has a much more highly developed type of parasitism than any of those in the preceding group, both as to host specificity and a restrained type of feeding which avoids immediate destruction of the invaded tissue.

c. *The Powdery Mildews.* The powdery mildews are caused by obligate parasites (Erysiphaceae) with well-developed haustoria that permit them to feed on the cell contents without extensive injury to the supporting tissue. The dense growth of mycelium and sporophores on the leaf surface accelerates respiration and undoubtedly decreases photosynthesis by screening out the sun's rays. However, infected leaves persist for weeks and may show only the slightest traces of necrosis. This could be attributable to the fact that relatively little of the parasite's body is inside the host tissue where its toxic secretions would be added to the cells of the host.

The specificity for hosts is very marked. The physiological races that differ in varietal host preference (which were first encountered in the more specialized leaf blights such as *Colletotrichum* spp.) are found regularly in the powdery mildews. The significant feature about the parasitism of the powdery mildews is that the parasite has no saprophytic existence apart from its host and must depend entirely upon specialization in parasitism for its survival. With this change, there appears some evidence of a very compatible establishment in the host even though the host tissue is not stimulated to excessive growth.

d. *The Rusts.* The rusts are caused by parasites (Uredinales) that are very similar to the powdery mildews in their parasitic ability. The two might be grouped together as obligate leaf parasites except that the rusts are even more highly specialized. In addition to physiological specialization of races for certain crop varieties there often is further specialization of the haploid and diploid stages for entirely different families of host plants. Heteroecism has been established as an essential aspect of the life cycle in many species. One can only conjecture as to how or why heteroecism came into existence and is actually obligatory for most of those species of rust which have no autocyclic stage.

The rusts have achieved remarkable progress in approaching a commensal relationship with the host. A hypersensitive reaction of the host cell that ends in its death is actually an immune reaction in this type of establishment. The more adept races of rust invade the tissue intercellularly without causing such injury. The haustorium or occasional intracellular mycelium approaches the nucleus or else attracts the nucleus to it so the two often adhere closely to each other. The parasite may be suspected of either finding special nutrients at this center of cell activities or else stimulates host metabolism from this location.

The susceptible host cell enlarges rapidly and begins to undergo division so a new type of metabolically active tissue is formed that is composed of the fungus and host cells in a very compatible relationship. There is a change in the rate of respiration and in the respiratory quotient indicating that a new type of metabolism has come into existence. This is different from normal host activity (Samborski and Shaw, 1956). No one knows exactly what transpires but there is a definite change in the citric acid metabolism of the tissue and there is strong indication that succinic acid affects the respiration in uredospores of *Puccinia graminis* (Staples, 1957a, b). As the rust pustule ruptures the cuticle to expose uredospores or spermogonia, there may be considerable necrosis of the host cells at the base of the lesion in the more resistant varieties and there is an appreciable loss in the water economy of the host.

In most susceptible species the leaf tissue remains active and functional while the rust spores are developing. A gall may be produced when infection occurs in cortical meristematic tissues such as those of the stem of red cedar parasitized by *Gymnosporangium juniperi-virginianae.* In such disease establishments the parasite has been able to regulate the host cells, without otherwise injuring them, to such an extent that the pathogenesis could very readily be classified in the sixth (following) type of parasitism. This type of pathogenicity is preeminent in such rusts as *Peridermium* spp. on pine but tendencies in this direction are to be found throughout the Uredinales.

6. *Diversion of Foodstuffs to Abnormal Uses*

The interference with utilization of food materials is encountered in many types of parasitism such as those discussed above in the rust fungi. Certain types of pathogens have the ability to invade the host cell either directly or by means of haustoria and alter its metabolism most drastically. Such tissues are stimulated to abnormal uses of foodstuffs in synthesizing materials that cannot be of value to the host, since they contribute nothing to the host's essential functions. There are three conspicuous groups of parasites in this classification: the smuts, gall-forming parasites, and viroses.

a. *The Smuts.* The smuts would seem to belong in the class of gall diseases because the parasites, with very few exceptions, convert host tissue into tumors, then assimilate the tumor tissue. The physiology of corn smut caused by *Ustilago zeae* is representative of this class. The chlamydospores produce sporidia that lodge in a leaf whorl, complete their sexual process, and invade the tissues by direct penetration. The mycelium becomes somewhat systemic as it infects nearby axillary buds and flowering organs before the internodes elongate. The various invaded tissues are not converted into smut galls until there is a generous supply of nourishment, according to Davis (1936). Because of this, galls are most commonly observed on the ear and lower portions of the stalk to which carbohydrates have been transported. The smut mycelium grows with the stimulated host tissue and eventually produces a mass of chlamydospores.

The process in the floral smuts is very similar to corn smut even to the point of the mycelium growing in the vegetative organs for relatively long periods without causing growth or necrosis in such diseases as bunt caused by *Tilletia tritici*. The state of restrained parasitism may endure for weeks or even months before the parasite becomes aggressively pathogenic. Pathogenesis awaits the proper stage of nutritional development in the host which, in this case, apparently occurs when the leaves begin to supply food materials to the young ovary. The food materials that would have enriched the endosperm and developed an embryo are diverted into nourishing the parasite and overgrown gall tissue.

b. *The Galls.* The gall diseases are incited by bacteria, lower fungi in the Chytridiales, and other fungi. The parasite apparently thrives by stimulating host metabolism. There is a richer pool of metabolites from which it can draw materials for its own growth and reproduction. As mentioned previously, the nodules produced on legumes by *Rhizobium leguminosarum* constitute a form of parasitism that definitely approaches commensalism.

The overgrowths caused by the crown gall and hairy root bacteria (*Agrobacterium tumefaciens* and *A. rhizogenes*) are special examples of this type of pathogenesis (Braun, 1947). The bacteria may multiply inside the host tissue without causing tumors but once a wound is created in the presence of growing bacteria the host cells proliferate a neoplastic tissue. The host must be exposed to the bacteria within a few hours after the wound is created but the bacteria may be eliminated later without jeopardizing tumor development. Under natural conditions, the bacteria may be restricted to the surface of the gall far removed from the site of hyperplasia. The exact nature of the growth incitant produced by the bacterium has not been determined but it is known to be thermolabile (Braun and Mandle, 1948) and the suggestion has been made that it is a polypeptide or nucleoprotein.

The principal feature of this gall development is that it seems to be definitely autocatalytic once the initial reaction is induced by the bacterium. The diseased tissue may be isolated in pure culture (White and Braun, 1941), separate from the bacteria. The aseptically grown tissues will induce a new gall when implanted in tissue of the host. This disease is almost unique in the annals of plant pathology in that the casual agent soon becomes incidental to the development of the disease. The processes of pathogenesis are autocatalytic and independent of parasitism after the initial period of induction.

There are several overgrowth diseases caused by obligate parasites related to the Chytridiales. These fungi penetrate a root either by way of the root hair or other tender epidermal cells or through a wound. Some produce an intracellular protoplast and others, the mycelial forms, send a haustorium into the cell. The host cell responds by enlarging and dividing. The naked protoplast in the host subdivides and continues its growth in the two new daughter cells so there is essential compatibility between host and pathogen.

Rather extensive studies have been made on the nutrition of crucifers affected with *Plasmodiophora brassicae*. For many years increasing the soil pH to 7.2 with lime was recommended as a preventive (Chupp, 1928) but there is good reason to suspect that the effect of the calcium ion was about as important as hydrogen ion concentration becauses the balance of calcium and potassium in the host cell will determine the severity of infection (Pryor, 1940; Gries *et al.*, 1944). Abundant supplies of potassium increase gall development and it has been shown by Palm and McNew (unpublished data) that potassium accumulates in the club tissue to such an extent that the normal growing points of the plant may be starved into inactivity. The potassium is essential for synthesis of inorganic nitrogen into amino acids and, undoubtedly, tissue rich in amino acids will promote the growth of both tumor cells and the plasmodium of the parasite.

c. *The Viral Diseases*. The viruses divert essential amino acids and nucleotides into synthesis of virus nucleoprotein. Insofar as known, these nucleoproteins can never be digested or converted into host protoplasm again even when the normal host tissue is being starved for nitrogen (Spencer, 1941). This loss of organic nitrogen to virus multiplication constitutes a drain on the host's metabolic processes.

There is much more to the pathogenic activities of the virus than the drain on nitrogen metabolism by the mere synthesis of nucleoproteins. A plant may support virus multiplication without any appreciable evidence of disease reaction. The most striking example is the so-called healthy or X virus of potatoes. It is essentially noninjurious to growth or physical appearance; but it accentuates the injury by other viruses in a synergistic fashion and it is virulent when transferred to other hosts.

This situation reminds one of the acquired resistance of tobacco for the ring spot virus. As described by Price (1932) and others, the established young leaves on an inoculated plant show the typical chlorotic oak-leaf pattern but leaves developed subsequently appear normal green. If the leaf primordia while still in the embryonic tissue are preconditioned by the virus, they do not produce symptoms even though the virus multiplies readily in them and is capable of causing symptoms on a new, unconditioned host.

It is now known that higher plants may synthesize heavy molecules of protein such as those resembling the tobacco mosaic virus (Takahashi and Ishii, 1952) without becoming diseased. The protein becomes viruliferous only when carrying the proper nucleic acids. One is forced to conclude that ability of the nucleoprotein to induce a disease reaction apparently resides in some secondary reaction not directly associated with abnormal synthesis of proteins.

There is one mechanism of pathogenesis by viruses that seems to be rather obvious. Many of them are known to interfere with the translocation of synthesized food materials. Phloem necrosis is very conspicuous in some of the virus diseases (curly top of beets and net necrosis of potatoes) and undoubtedly the presence of large molecules in the phloem tubes increases the viscosity of the phloem sap and may interfere with its passage through the sieve plate.

As shown by Bennett (1932, 1937), viruses move in the direction of translocation of synthesized foodstuffs to food-deficient areas. Therefore yellow mosaic virus in raspberry, for example, moves downward into the crown in the fall and upward into the young, actively growing shoot in the spring. Any interference by the virus would lead to inefficient metabolism as carbohydrates accumulated in the leaves or storage organs and could not be moved expeditiously to the growing tip or into storage as required. These interferences with movement of foodstuffs could justify a separate classification for some viruses but this has not been done because so little is known about the real nature of their pathogenesis either in phloem or parenchyma cells.

B. *Resumé of Natural Processes of Pathogenesis*

This brief resumé shows that there is a rather general correlation between the type of parasitism and the physiological function of the host that is interrupted. The causal agents range from facultative saprophytes, in which parasitism is incidental to their ordinary activities, to obligate or facultative parasites with commensal tendencies. Not much in the way of physiological specialization is required of a fungus or bacterium that digests a food reserve when it happens to lodge in a wound through the protective covering. Even other forms of tissue that are actively growing may be destroyed by parasites that are only slightly more specialized.

However, when parasites begin to adjust their activities to special tissues such as the tracheal tubes or to send their cells into a living host without digesting the cell or otherwise poisoning its activities, they must have a tight control of their pathogenic activities. Finally, if the parasite in the cell supervises and directs the activities of that cell so that its own multiplication is enhanced, it must produce growth-regulating chemicals that induce new reactions or retard differentiation of cells into tissues. The alteration of host activities to the benefit of the parasite may be considered as an ultimate development in parasitism because it is only one step removed from commensalism in which two subjects share the same pool of metabolites without injuring each other. Few parasites, however, make a unique contribution to the partnership which would enhance the strength and vitality of the host so that the symbiotic state would be definitely superior to independent existence for both members.

As one progresses through this series of physiological disturbances caused by parasites, he becomes aware of certain basic changes in their habits. There are three well-marked trends. The first is suppression of a tendency to destroy cells by cytolytic enzymes. The second is a progressive change from indiscriminate general invasion of all cells to establishment between cells or intracellular penetration with haustoria to feed inside the cell while the major body resides outside and, finally, to a true intracellular existence where proteins of host and pathogen are freely associated. The third change is the growing tendency for the host cell to respond actively to the parasitic establishment and for the parasite to regulate this response. However, the parasite sacrifices autonomy when it prolongs and expands a favorable activity in the host.

The concepts of this section are based upon the assumption that there must be some set of basic principles behind pathogenesis. These seem to emerge as one begins to classify the physiological effects involved. If there is much validity to this arrangement, it should be possible to reinforce its concepts by interpreting, in its light, such things as the evolutionary processes that are operating in pathogenesis, and the response of the diseased state to changes in the nutrition and heredity of the host.

IV. The Evolution of Parasitism and Pathogenicity

From a strictly teliological viewpoint, a parasite has very little to gain from unbridled exercise of pathogenicity. If food procurement is so destructive that the host is eradicated, the parasite usually will have defeated its own purposes in life. The more compatible it can be with the host cell, the better are its chances of propagating itself indefinitely.

There is little advantage to be gained from making a plant body into a corpse that can be overrun by every saprophyte in the neighborhood.

Can evidence be found that parasitic specialization does improve and progress toward a state of mutual tolerance as outlined in the preceding section? Would it be possible for a soil saprophyte that ordinarily survives by digesting miscellaneous organic matter to become adapted by evolution to a specific host, to specialize its attack on certain tissues of the host, eventually to forsake the freedom of saprophytism and become so specially adapted to its host that it depended entirely upon it?

There is no ironclad evidence of such complete evolution, but anyone who has worked with plant pathogens knows that their virulence is a tenuous thing. Pure cultures usually become avirulent when maintained for long periods on culture media generously provided with all nutrients necessary for growth. There is ample evidence that new parasitic races may arise in culture under aseptic conditions or in the host through somatic mutation or segregation of factors for pathogenicity.

Fortunately, there are some observations that phytopathogenic bacteria may be arising under field conditions from saprophytes that first acquire parasitic ability and then proceed to develop chemicals to promote their pathogenesis. Furthermore, there is excellent experimental evidence on other species that host and parasite eventually establish a tolerable level of parasitism in which host and parasite can coexist. Unfortunately there is no comparable evidence of how parasites forsake saprophytism entirely or assume a compatible conjunctive relationship.

A. *The Tobacco Wildfire Group of Bacteria*

The wildfire of tobacco is caused by a green fluorescent bacterium known as *Pseudomonas tabaci*. Whenever weather conditions favor the dispersal of this pathogen by driving rain while the leaves are water-soaked, epiphytotics erupt with explosive damage. Somewhat less virulent in its effects on tobacco is a second pathogen known as *Pseudomonas angulata*, the cause of angular leaf spot. In addition to these two species, there is a third green fluorescent bacterium present in tobacco fields. It is the very widely distributed *Pseudomonas fluorescens* that is a general soil saprophyte in many areas both in the tobacco growing region and elsewhere. These species are normal soil inhabitants that multiply profusely in the vicinity of wheat and grass roots where they apparently find nourishment from root exudates (Valleau *et al.*, 1943).

There is good reason to suspect that these three species are variant strains of the same thing in spite of their different names. The only real substantial reason for giving them separate names is that they differ in pathogenicity. If the soil saprophyte *Pseudomonas fluorescens* is sprayed on a tobacco leaf, it does not invade the tissue. However, certain strains isolated from healthy-appearing leaves, will infect and cause lesions on weakened plants (Reid *et al.*, 1939). If *Pseudomonas angulata* is sprayed on a plant whose leaves have been preconditioned by exposure in a moist chamber, a necrotic spot is formed ranging from a minute speck less than 1 mm. in diameter to an angular spot bordered by veins surrounding the locus of invasion. *Pseudomonas angulata* is a reluctant parasite that invades leaf tissues readily only when they have been water-soaked. There are strains of this bacterium that are absolutely non-aggresive and will not invade the leaf tissue or cause lesions. These avirulent strains soon come to predominate in a culture of the pathogen maintained on nutrient agar. To all intents and purposes they are identical to *Pseudomonas fluorescens* in cultural and physiological characteristics (Reid *et al.*, 1942).

The wildfire organism causes a larger lesion than *Pseudomonas angulata* and it usually is bordered by a chlorotic halo ranging from 5 mm. to 2 cm. in diameter. This halo is due to the production of an exotoxin by *Pseudomonas tabaci* that has been identified as a new *a*-amino acid by Woolley *et al.* (1952a, b) who gave it the name tabtoxinine. This toxin weakens the tissue and predisposes it to more extensive invasion by the pathogen. Variant strains can be isolated from pure cultures of *Pseudomonas tabaci* that do not produce toxin. They are identical in all respects with *Pseudomonas angulata.* There can be little doubt that these two so-called species are identical because they have identical cultural characteristics, morphology, serology, and physiological properties according to Braun (1937).

Either with or without the toxin, the *Pseudomonas tabaci* complex is a poor parasite. The host must be preconditioned to infection by water-soaking the leaves as described by Clayton (1936). Leaves exposed to extreme root sap pressure from wet soils or water-logged by prolonged driving rains may be invaded extensively, particularly if the tissues have been weakened by inadequate supplies of potassium during their growth.

Thus we have in the tobacco fields of Kentucky, Pennsylvania, and undoubtedly elsewhere a complex mixture of green fluorescent bacteria. The omnipresent soil saprophyte is encouraged to grow by exudates from the roots of wheat, barley, and other plants. Undoubtedly if enough of them splash onto the lower surface of tobacco leaves injured by inclement weather, an occasional one might multiply (Reid *et al.*, 1942). After two or three host passages it should become aggressive enough to cause angular lesions on weak plants. Those strains that acquired the ability to produce toxin would have much more serious pathogenic abilities. The sequence of changes is known to occur in reverse order so it is logical to assume that increase in virulence could be developed as outlined here.

B. *The Bacterial Wilt of Maize in Resistant Hosts*

Parasitism has not evolved beyond this stage in wildfire. The parasite is still a facultative saprophyte that destroys the tissue indiscriminately whenever the host is weakened by unfavorable weather and soil conditions. The next stage of progress can be illustrated by well-documented experiments with the sweet corn wilt bacterium (*Bacterium stewartii*) which shows a primitive ability to adjust its activities to its host.

This bacterium also produces variant strains in culture that may be either more or less virulent than the parent cell. One weakly virulent strain was found to have lost its ability to use inorganic nitrogen. Since the nitrogenous materials of the tracheal sap are primarily inorganic in nature, the loss of ability to reduce these materials to organic forms would handicap the survival and growth of the bacteria (McNew and Spencer, 1939).

When such weak strains are inoculated into the host in massive dosages, they rarely cause infection, but small chlorotic lesions may occur on an occasional leaf. The bacteria from such lesions will progressively gain in virulence as they are transferred from plant to plant. Eventually they become fully aggressive. These virulent progeny from avirulent strains simultaneously regain the ability to assimilate inorganic nitrogen. The proper interpretation of these data is that the avirulent bacteria have all the attributes necessary for virulence except ability to use the primary nitrogen sources available in the host tissue, and once the bacterium has developed the proper enzymes to reduce nitrate and ammonium ions, it attains a full complement of parasitic abilities.

It follows that if any progeny of this strain should acquire the ability to use inorganic nitrogen in culture it should be more virulent in the host. This was demonstrated in two types of experiments (McNew, 1938). When massive transfers were made from peptone agar to a mineral nutrient agar containing only inorganic nitrogen, an occasional colony began to grow. After several transfers on the inorganic nitrogen medium to fix this attribute firmly, all of the new cultures were found to be fully virulent for sweet corn. This demonstrates that organisms may gain virulence while in pure culture under the proper conditions.

A comparable evolution of parasitism was obtained on organic nitrogen medium by mechanically segregating variant strains as they appear by dispersing the cells in peptone-beef agar dilution plates, isolating the colonies, and testing subcultures of each for virulence. The process of selection was repeated with the most virulent member. After four such mechanical selections, a fully virulent strain was isolated from cultures maintained constantly on peptone agar. The virulent strain had concomitantly gained ability to assimilate inorganic nitrogen. The simultaneous restoration of virulence and ability to use inorganic nitrogen was due to selection of natural mutants rather than to development of adaptive enzymes since the parent avirulent type and its progeny had never been exposed to either the host environment or inorganic nitrogen in medium before the final test for virulence was made.

Undoubtedly there are dozens of attributes such as ability to use inorganic nitrogen that are prerequisites for parasitic establishment in the tracheal tubes. The resistant varieties of maize may be suspected of having some specific difference in physiology that prevents full parasitic development. If so, bacterial cultures injected into hosts with different resistance would be exposed to different selective environmental pressures. When a weakly virulent culture is injected into a susceptible host it ordinarily gains in virulence as is witnessed by the example just discussed; but would the highest state of virulence be attained in the most susceptible variety of maize? When Wellhausen (1935, 1937) investigated this by making serial passages through resistant and susceptible inbred lines of maize he found that the bacteria from the more resistant lines were more virulent than those from susceptible lines when tested for virulence on the same host. Extensive tests with several different cultures prove that each of these in a given line of maize attained a comparable maximum degree of virulence after nine or ten serial passages and remained constant thereafter depending on the innate resistance of the host. Wellhausen (1937) and Lincoln (1940) were able to demonstrate that the more virulent cultures from resistant lines were readily attenuated by serial passage through a more susceptible line. Irrespective of initial virulence of the culture, they came to have essentially identical ability to cause wilt when maintained in a host of specified resistance.

C. *Variations in Michigan Wilt of Tomato*

There is a limit to the operation of this principle of host-regulated virulence. When different species of hosts for *Corynebacterium michiganense* were inoculated with a highly virulent culture isolated from *Hyoscyamus niger*, virulence changed very slowly (McNew, 1938). Subcultures transferred serially in very susceptible hosts such as *H. niger*, *Nicotiana glutinosa*, and *Lycopersicum esculentum* maintained a high degree of virulence without developing physiological specialization. The same culture maintained in very resistant *Phaseolus vulgaris* gained only slightly in virulence for this host while losing some of its virulence for the other hosts. Thus the principle laid down for *B. stewartii* in inbred lines of maize does not apply to *C. michiganense* in different species of plants. This is in general agreement with Wellhausen's observation (1937) that *B. stewartii* does not gain in virulence for maize when cultured in resistant grasses.

The data on *B. stewartii* are probably indicative of what goes on in nature over a long period of time. As deduced by Zinsser and Wilson (1932), the rise and fall of epidemics may be determined by dissociation of the causative agent to give less virulent strains. There is no evidence that virulence increases indefinitely among animal hosts and eventually the less virulent variants will dilute the inoculum potential of a culture. There are good statistical reasons why these weaker forms eventually would reduce the severity of an epidemic.

There are two divergent influences in the epidemic of a plant parasite that could make its course differ from that in animals. There is very little evidence that antibodies develop in plants to give a true immune reaction as in animals. The nearest approach to this is the immune reaction or recovery of certain plants such as tobacco from ring spot virus as discussed in the preceding section.

The second major difference between animal and plant diseases is that the health of the individual plant is of less concern than the fate of the entire population. Population genetics change more rapidly in plants so the natural laws of selection and adjustment to epidemics are more obvious as described below.

V. The Law of Host-Parasite Balance in Pathogenesis

The host population in most species of plants has a diversity of genetic material. In the natural state this provides plants that are likely to differ in their reaction to parasites. In the course of a severe epidemic, the more susceptible members are destroyed or so seriously handicapped that they do not reproduce. This automatically increases the resistance of the entire population for that particular disease.

The end result is that the host may become adjusted to each change in virulence of the parasite. This means that the host and parasite establish a balance or coexistence at a higher level of parasitic tolerance for each change in the parasite unless there is a mechanism for attenuating the parasite as described above for *Bacterium stewartii.* Undoubtedly if there is a loss in virulence, more susceptible types of host would survive and the host-parasite balance would be established at a lower level of parasitism. This process of readjusting resistance and virulence to a balanced state might be designated as the natural law of survival in pathogenesis.

A. *The Races of Wheat Rust*

The existence of this law can be demonstrated from studying some of the things that man has done to change the host-parasite balance. Much has been learned about what can be done—and what should not be done—in changing the natural balance that parasites and hosts have been able to achieve over the centuries. An excellent model for demonstrating the law may be seen in the breeding of wheat for resistance to *Puccinia graminis* in the United States.

The stem rust susceptible varieties of 1910 such as Bluestem and Fife were replaced by the rust-escaping Marquis. The rust epidemic of 1916 drove farmers to replace Marquis with the durum wheats. These in turn were susceptible to several races of rust of which No. 11 was outstanding. A cross of the varieties Marquis by Kota produced the variety Ceres which was immune to the races of black stem rust prevalent from 1926 to 1934. However, a new race designated as No. 56 which would attack Ceres was identified as early as 1928 and became prevalent by 1934. It reached severe proportions in 1935 and 1937 and sealed the fate of the variety Ceres. Losses of 160,000,000 bushels of wheat occurred in the United States during 1935.

Ceres was replaced by the variety Thatcher which had withstood the epidemic of race 56 in 1935. Thatcher, Hope, and hybrids of them were widely used in subsequent years. These varieties contained a combination of genes for resistance derived from varieties of durum, emmer, and flour wheats which was adequate to control the prevalent races of stem rust (17, 19, 38, and 56). Race 15B was discovered in 1939 and gradually increased in the next few years. Its biotype 15B2 swept over much of North America in 1950 since it could attack resistant durums. By 1953 and 1954 the macaroni wheat varieties had been virtually annihilated and the plant breeders had to turn their attention to use of resistance obtained from varieties in Kenya.

In looking back over the past 50 years, one is impressed that the germ plasms of *Puccinia* and *Triticum* have been playing tag with each other. As soon as a new set of genes for resistance was introduced into the crop, the parasite developed a new race for virulence. In only 20 of the 50 years between the great epidemics of 1904 and 1954 was wheat adequately protected from *Puccinia graminis* on the great plains of North America according to Stakman and Harrar (1957, p. 507).

At each step along the way, man speeded up the processes of evolution by seeking out the sources of resistance around the world and introducing the genes into the commercial crop. Very shortly a new physiological race of the parasite would come into prominence and restore the parasitic balance in favor of the pathogen. Then the plant breeder reacted, and so on, *ad infinitum*. Unfortunately for the crop, its custodian runs a poor second in this race because rust operates on a 14-day cycle and the breeder on a 7- to 10-year cycle for propagation of a new variety.

Plant parasites that get out of control and violate the law of natural balance are the exception rather than the rule. Usually this has occurred in modern history (the past 200 years) only when man has been primarily at fault. Unfortunately men are careless and often prone to make mistakes. There are four common blunders against the law of natural balance in pathogenesis which set the stage for devastation by plant parasites.

B. *The Role of Introduced Parasites*

The most serious offense is the introduction of a virulent parasite into an unconditioned population of hosts. If the population has never had an opportunity to participate in the evolution of pathogenesis it is completely at the mercy of the parasite. We need to cite only a few examples. The introduction of downy mildew of grape caused by *Plasmopara viticola* into the vineyards of Medoc, France, from the United States was a national catastrophe. The importation of chestnut blight caused by *Endothia parasitica* into the United States, presumably from China, deprived an entire nation of one of its most valued forest trees. Never has a pathogen created such havoc on a defenseless population over such an acreage of land. About 50,000,000 acres of chestnut forest were eradicated because resistance of the host had not evolved to the same level as the parasitism as its enemy.

The citrus industry of this country escaped a comparable ravage from citrus canker caused by *Xanthomonas citri* only by taking drastic action when it was introduced from the Orient. Groves extending over thousands of acres had to be burned in order to eradicate the parasite. The downy mildew of hops caused by *Peronospora humuli* was introduced into England from Japan in 1917. Within 5 years, it became the limiting factor in hop culture in western Europe.

C. *The Attack on Introduced Plants*

A second mistake that man has made repeatedly in upsetting the disease balance in crops is to introduce a new crop into an established population of parasites. Although the results are not so disastrous economically as the reverse situation, the results are no less sensational. The American colonists soon found that their favorite table and wine grapes from Europe would not grow in the New World. The logical assumption was that they were not adapted to the climate. Today we know that they were wiped out spontaneously by the downy mildew disease which went to them from the native wild grape.

A similar situation was encountered when the superior varieties of *Hevea* rubber trees were introduced into South America from the Orient. They were quickly eradicated by the leaf blight fungus, *Dothidella ulei,* that was indigenous to this area. In the 75 years that *Hevea* had been cultivated and bred for better yield in the Orient, it had lost all resistance to this pathogen. The wild types in Brazil, from which the new varieties had been derived, had maintained their resistance because they had been exposed continuously to infection pressure and natural selection.

When the American colonists settled along the Atlantic seaboard in the 17th century they found that the wild apples, crabapples, and hawthorns were infected with fire blight caused by *Erwinia amylovora.* According to the accounts by Cox, the trees on the banks of the Hudson had mild infection that caused the loss of an occasional branch but the disease seemed to fluctuate around a mild endemic level. However, when the horticulturally superior pears of France and Western Europe were introduced, they were literally eradicated. As men began to assemble select varieties of apples into orchards where the disease could spread easily, fire blight became a chronic problem. Evolution of the parasite continued while man held the evolution of the host static under conditions ideal for infection.

Apparently as long as *Malus* spp. and other rosaceous hosts were distributed in a general plant society, *Erwinia amylovora* was restricted in its spread. Some trees were undoubtedly destroyed but the more tolerant ones escaped destruction and reproduced their kind of resistant progeny. If a virulent new strain of the bacterium arose, its spread was hampered sufficiently by mixed plantings to prevent eradication of the hosts before a new type of resistance could be evolved. Thus the host and parasite had come into a normal balance before man upset the scheme of things by introducing new hosts that had never been exposed to natural selection by infection and trees were crowded into orchards where conditions favored the parasite.

D. *The Aftermath of Inadequate Plant Breeding*

Man also upsets the balance of resistance-pathogenicity by efforts to breed superior crops. For many years the unscientific horticulturist selected new varieties for yield, quality of produce, and ecological adaptability. If pathogens were not present so that varieties would be chosen automatically for disease resistance, the breeder could, and often did, lose all innate disease resistance just as the breeders of rubber trees did in the Eastern Hemisphere.

There was enough heterozygosity left, however, in the open-pollinated field crops to keep the new varieties from being exposed to the full force of epidemics. Any infection on a susceptible plant might be checked or handicapped by the more resistant members in the mixed population. Often this was not true in the vegetatively propagated fruit crops; so diseases of orchards, rubber, banana, and fruit plantations became uniformly destructive and demanded special disease control measures. The breeders of field and vegetable crops were somewhat slower in reducing their crops to completely uniform, susceptible populations. They had to learn how to develop pure lines by inbreeding and subsequent hybridization in order to make every plant in a field uniformly susceptible or uniformly resistant.

In the period 1890–1910, Orton and others demonstrated that varieties could be bred for resistance by selection and hybridization. This is one of the greater gifts to the welfare of mankind. After the force of hybrid vigor had been exploited in the era 1930–1940, it was obvious that the greatest remaining improvement which could be made in many crop varieties was to improve their resistance to disease.

This has not proved to be as easy as it sounds. As indicated by the story on breeding wheat for rust resistance, the plant parasites are versatile adversaries, and much more skill is required than simple hybridizing and backcrossing. In some instances serious problems have arisen from breeding for disease resistance because uniform resistance to one parasite may mean uniform susceptibility to another one.

E. *The Victoria Oat*

This is well illustrated among the otherwise remarkable achievements during the past 30 years in breeding oats for resistance to crown rust caused by *Puccinia coronata*. By hybridizing and selection, the old unimproved varieties were given stem rust resistance from the varieties White Tartar and Richland and smut resistance from Black Mesdag. In order to cope with the changing population of crown rust, the resistance of Victoria was bred into this synthetic variety. The superior varieties obtained from this cross swept into popularity and dominated oat culture in the United States during the period 1942–1948.

The genes from Victoria induced a hypersensitive reaction to invasion by all biotypes of *P. coronata*. As soon as the leaf tissue was invaded, the adjacent cells died and the infection was destroyed before spores could be formed. Host injury was restricted to a small fleck. This reaction which is equivalent to immunity apparently set the stage for the development of the seedling blight caused by *Helminthosporium victoriae*. This fungus was a minor parasite on grasses and it had never attacked enough oats to attract attention. However, it is a facultative saprophyte that thrives on dead tissue, so, apparently, the hypersensitive reaction of Victoria-like varieties promoted its parasitism. The *Helminthosporium* blight gained momentum and within 3 years after the fungus was named it had become a major factor in oat production. Yields were being reduced by 25 to 50% wherever weather conditions were favorable to fungous development.

Fortunately, other forms of resistance to crown rust were available in the variety Bond. New varieties were developed to replace the Victoria type within 5 years but, as predicted by H. C. Murphy, the varieties were soon attacked by a new race of rust that came into prominence as soon as the Bond varieties were used extensively. These in turn were soon replaced by new varieties that had derived their resistance from the varieties Sante Fe and Landhaufer.

Nowhere in the annals of plant pathology is there a better illustration of the evolution of pathogenicity toward a nominal host-parasite balance than in this story of man's struggle with the fungi for the oat crop of North America. We have witnessed in 3 decades a process by which man speeded up evolution many hundredfold.

Thousands of years might have elapsed while germ plasm of host was matched against germ plasm of the parasites had not man intervened. He brought an assortment of oat germ plasm from Australia, South America, Russia, etc., into an arena where the two types of parasites could operate freely. As soon as one gained ascendancy over the host, the variety was removed before it was eradicated. New germ plasm was introduced just as would have been done inevitably, but much more slowly, under natural conditions.

Man's role was to create ideal conditions for epidemics by planting millions of acres of oats tightly packed with uniformly resistant—and uniformly susceptible—plant material. The pathogens were uniformly handicapped at some times, and uniformly successful at others. New germ plasm was fed into the arena as it was needed. In short, man was forced to speed up the processes of evolution to maintain his food supply in this generation. At present he has everything under control but no one knows how long it will be before the next stage of evolution will present itself.

The lesson to be derived from this experience is that the art of breeding for disease resistance depends more on an understanding of pathogenesis and the nature of parasitism than it does on genetic manipulation. There is nothing so profound in the processes of inbreeding, hybridizing, and backcrossing that any technician could not master them in a few months. The real problem is to estimate what course evolution of the parasite is likely to follow and what hazards of pathogenicity are likely to be encountered after every genetic change in the host.

This brief account of the evolutionary processes by which parasites come into balance with host resistance and the disasters to which man has exposed his favorite crops by meddling, makes one wonder whether it is safe to breed forest trees for resistance. Until much more is known about the nature of their diseases and the genetic potentials for evolution of their parasites, extreme caution must be observed.

What might happen, for example, if every white pine tree in the United States was uniformly homozygous for resistance to *Cronartium ribicola?* If a new race of white pine blister rust arose spontaneously, every tree might be uniformly susceptible. The disease would sweep across young forest plantations like wildfire wherever there are wild currants. At least 75 to 125 years would have to elapse before a new resistant line could be developed and put into commercial production. The disaster to an annual crop such as oats described above would be nearly fifty times as difficult to correct technically and probably would be even more serious economically for forest trees.

The least that should be done is to forbid widespread planting of any one line of forest trees. Blocks or mixed plantings of trees with different hereditary attributes should be used. Furthermore, there is great need in breeding trees to find the best possible means of transferring one or two genes for resistance without reducing genetic heterogeneity for other attributes in any way.

VI. The Effect of Environment on Different Classes of Parasitism

By classifying plant diseases into the six physiological classes according to their effect on the host, an elderly arrangement of plant diseases has been obtained. It is obvious from the discussion in the preceding section that certain forces operate to cause an upgrading of parasitic ability. There are very substantial data to prove how the parasitic way of life can be developed from saprophytism to give representatives of the three lower classes of pathogens. There is also appreciable circumstantial evidence of evolution in the higher classes in which the forces of parasitism and host resistance can be kept in balance even as virulence increases or decreases.

The physiological basis of classifying diseases should be valuable in understanding how environmental changes would influence the balance between host and parasite. The changes in soil and air would be expected to affect the different classes of parasites differently because their basic relationships to their respective hosts are different. Those parasites that specialize in a saprophytic existence apart from their hosts would be more directly affected by the change while those that are highly dependent or obligately dependent upon the host might be affected more indirectly through the host's response.

Those parasites that prosper on very actively growing, metabolically active cells should be most severe on the well-nourished vigorous plants. On the other hand, the facultative saprophyte that attacks only the injured or weak plant might be expected to be most destructive on plants grown under adverse conditions, particularly if those conditions were also conducive to its own growth and multiplication on or near the host. A brief review of the effect of weather and soil fertility will serve to substantiate these ideas.

A. *The Effects of Weather*

The facultative saprophytes that cause the first three classes of disease (the storage rots, the seedling diseases, and the root rots) are almost entirely dependent on the environment. A modest change in the ventilation or temperature of an apple or sweet potato storage will prevent *Penicillium* rot and black rot infection, respectively. Only a slight increase in soil moisture content is necessary to cause pea seed decay by *Pythium ultimum*. If there is free water in and on the soil for a day or two, the emergence of seedlings may be reduced 60% or more, but if the moisture content remains at about 30 to 50% of water-holding capacity, a perfect stand will be obtained. Lima bean seed, on the other hand, are destroyed much more readily by this same fungus if development of the seedling is handicapped by cold soils for a few days. A cool weather crop such as the pea is rendered more susceptible by water than by a drop in temperature while the reverse is true in the warm weather crop such as the lima bean.

The severity of *Aphanomyces* root rot of peas can be accentuated appreciably by reduction in soil temperature while the *Fusarium* root rot of the same crop can be very severe in warm seasons. Minor changes in the hydrogen ion concentration of soil will alter the survival and parasitism of such soil inhabitants as *Pseudomonas solanacearum* on potatoes and *Thielaviopsis basicola* on tobacco. The addition of nitrogenous materials to soil will reduce the survival of *Ophiobolus graminis*.

Many soil saprophytes are destroyed or inhibited by microbial antagonists that can be stimulated by addition of organic matter in the form of animal manure, green manure, or even sugar.

These various facts are well recognized and are commonly used in controlling the poorly specialized parasites that live in the soil. Every effort is made to reduce their incidence or to handicap their development by various means. Disease-escaping practices such as sanitation, crop rotation, planting on selected dates, and choice of fields that are unfavorable to the pathogen are widely recommended.

Analogous procedures are also used for some of the leaf blight parasites that are poorly specialized although less use is made of environmental control except to insert toxic spray materials into the scheme.

Comparable precautions are rarely emphasized in controlling the more specialized parasites of the leaf. The genetics and growth of the host determine the aggressiveness of the parasite. The viral diseases and rusts are exceptionally destructive on succulent, rapidly growing plants.

B. *The Effects of Mineral Nutrition*

The mineral nutrition of plants may have a marked effect on the severity of their diseases. Although few diseases are completely controlled by varying the soil fertility, the damage from the pathogen may be ameliorated appreciably by adjustment of the balance and total quantity of nutrient elements in the soil.

If diseases are considered *in toto* there seems to be very little logic to the effects of various nutrients when the data available in some 700 reports in the literature of this subject are brought together. For example, the reports on nitrogen supply show that a deficiency of this element decreases the severity of 19 diseases and increases it in 11. On the other hand, a generous supply of nitrogen decreases the injury in 32 diseases but increases it in 58 others. About the only conclusion that can be formulated is that excess nitrogen probably should be avoided but every disease has to be considered as a special case. The same confusion exists when the responses of various diseases to potassium or phosphorus are listed.

If, however, mineral nutrition is considered in terms of the six physiological classes of diseases, a reasonable degree of order can be established. There are certain effects from mineral nutrition because disease production by the parasite and recovery of the host are physiological processes dependent upon nutrition.

Usually there are certain balances in nutrient elements that dominate the disease response of the plant. For example, an increase in phosphorus supply in soils reduces the severity of the take-all disease of wheat caused by *Ophiobolus graminis* in Kansas and Australia while an increase of nitrogen often has the same effect in Canada and England (see review in McNew, 1953). The two elements must be balanced in order to promote quick recovery of infected plants. The type of nutritional balance required varies most remarkably with each class of pathogens as briefly summarized below.

1. *Destruction of Food Reserves*

The storage rots and decay diseases that destroy the food reserves, are caused by the facultative saprophytes, are only moderately affected by the type of soil fertility prevailing at the time the crop produced the fruit, seed, or other storage organs. However, plants supplied with an excess of nitrogen do produce a crop more susceptible to decay. Careful study on the storage rots of apples in Northern Ireland and New Zealand have shown that fruit from heavily manured trees are more seriously injured by fungal pathogens than from those grown on untreated trees. The fruits from nitrogen-fed trees are more nourishing to *Cytosporina ludibunda* as can be demonstrated by removing sections aseptically and inoculating them in culture dishes.

Moderate applications of potassium around apple trees produce a firmer fruit less susceptible to mechanical injury and consequently to decay but an excess of potassium promotes physiological breakdown of the tissues. The general conclusion seems to be that an excess of nitrogen renders the fruit more nutritious to the pathogens. The effect of nitrogen is strictly on the growth rate of the pathogen in the presence of an unlimited supply of carbohydrates since there is no evidence that the host enters into a counter-reaction or growth response of any sort. In this type of disease, nitrogen or nitrogen-potassium balance is the predominant factor in determining host invasion.

2 and 3. *Prevention of Seedling Metabolism and Procurement*

The seedling diseases and root rots are definitely affected by soil fertility. An excess of nitrogen increases susceptibility to invasion of the plant tissues by the soil inhabitants with low grade to average pathogenicity traits. Apparently an excess of nitrogen either injures the seed and seedling or prolongs the juvenile condition of susceptible tissues that normally become more resistant with maturity.

The balance between nitrogen and phosphorus is very definitely important in such diseases as take-all, foot rot, and *Pythium* root rot of wheat. The proper balance assures rapid regrowth of roots from the crown of infected plants (Garrett, 1948) so as to assure a quick recovery from loss of diseased roots. The phosphate fertilizers are no less important in reducing the infection of sugar beet seedlings by *Aphanomyces cochlioides* (Afanasiev and Morris, 1949). By speeding up growth so the seedlings can rapidly pass into the resistant stage, losses in stand are reduced and yields are increased threefold. A comparable effect of nutrient balance has been demonstrated for Texas root rot. One may conclude that nitrogen-phosphorus balance is important in seedling diseases and root rots because it permits maximum growth of roots and rapid recovery from these low grade but very destructive pathogens.

4. *Interference with Upward Transport*

The wilt diseases, that affect movement of water and nutrients on the other hand, are much more affected by the balance of nitrogen and potassium than by nitrogen and phosphorus. An excess of nitrogen stimulates the severity of wilting and a deficiency reduces wilting apparently because of the attendant succulence of the tissue and because a tracheal sap rich in nitrogen is a more acceptable culture medium for the pathogen (McNew and Spencer, 1939) than a sap poor in nitrogen. This situation holds for the fusarial wilts of cotton and tomato, the bacterial wilt of maize, and comparable diseases.

If the plant is provided optimum supplies of nitrogen, its susceptibility is decreased by additional potassium supplies. The most logical explanation is that adequate supplies of potassium must be available to promote the utilization of available nitrogen in the processes of host growth (Shear and Wingard, 1944). This prevents accumulation of extra nitrogen that could be quite useful in promoting the growth of the parasite in the tracheal tube. Phosphorus increases the severity of infection only slightly and essentially has no effect on wilting until it is provided in excessive supply as was shown for bacterial wilt of maize (Spencer and McNew, 1938).

5. *Destruction of Food Manufacture*

a. *The Leaf Blights, Cankers, and Rots.* The leaf blights and cankers are rendered more destructive by nitrogen fertilization except for a few examples such as the bacterial shot hole of peaches caused by *Xanthomonas pruni* where nitrogen retards abscission formation and helps prevent defoliation of infected leaves. There is also some skimpy evidence that nitrogen fertilization may prevent extreme severity of early blight on tomatoes infected with *Alternaria solani.*

The more common experience, however, is for nitrogen fertilizers to increase susceptibility to invasion by parasites such as *Erwinia amylovora* on branches, foliage, and flowers of pear and apple, or *Pseudomonas tabaci* in tobacco wildfire. The use of moderate supplies of nitrogen and adequate supplies of potassium leads to firmer tissue in leaves, stems, and fruits so these organs are less severely invaded. The outstanding example is the use of potassium to prevent or reduce the susceptibility of tobacco leaves to water-logging of tissues, a major prerequisite to extensive invasion by the wildfire bacterium.

c. *The Powdery Mildews.* Powdery mildew infection is handicapped by cells that have thick, tough cell walls. Because of this, plants are rendered susceptible by heavy fertilization with nitrogen (Stuch, 1926). As a general rule, plants receiving generous allotments of potassium and phosphorus are much less severely infected. Potassium is particularly important and a few investigators have reported that a combination of potassium and silicates speeds up the hardening of leaf cells so the mildew fungi are less invasive.

d. *The Rusts.* The rusts of cereal crops are more severe on plants supplied with nitrogen. Moderately resistant varieties of wheat, rye, and oats may be rendered susceptible by depriving them of potassium while their resistance may be increased by adding potassium to the nutrient medium according to German investigators (Gassner and Franke, 1934; Gassner and Hassebrauk, 1933). Gassner and Hassebrauk (1931, 1934) explain these effects by the changes in amount of albumen in the leaf tissues since this is increased either by addition of nitrogen or removal of potassium from the nutrient supply. However, this is probably only one facet of the problem, since wheat plants provided with additional nitrogen will produce a toxin for *Puccinia glumarum* at a more rapid rate than normal.

6. *Diversion of Foodstuff to Abnormal Uses*

b. *The Galls.* The galls are affected most conspicuously by the balance between potassium and calcium. There must be adequate supplies of nitrogen and phosphorus to support cell division in the tumor just as in normal tissue so a deficit of these materials will suppress the severity of club root of crucifers, crown gall of tomato, bunt of wheat, and common scab of potatoes. However, if these two nutrients are available in adequate supply for host growth, additional amounts have only moderate effects compared to potassium and calcium.

There has been much confusion about adding lime to soils for club root control and use of acid-forming materials for potato scab because attention was focused on soil reaction rather than the change in nutrient balance in the host. The hydrogen ion concentration of the soil solution is not the sole factor operating because severe club root of cabbage may occur in soils at pH 7.0 to 8.2.

The amount of calcium ions available and, even more, its balance with potassium determine the severity of the growth response to invasion. Contrary to what was observed in the wilt and rot diseases, an increase in supply of potassium promotes gall growth. As a matter of fact, a large club on the main taproot of a cabbage or turnip plant will accumulate so much potassium that the remainder of the plant may be deprived of supplies adequate for normal growth. This situation is readily understood when it is realized that the potassium is indispensable for conversion of inorganic nitrogen into amino acids. The calcium is necessary for spindle formation and other processes involved in cell division and in the formation of new cell walls. Again there is a very sound reason why this particular nutrient balance has proved so consistently important when the nature of the pathogenesis is given full consideration.

c. *The Viral Diseases.* In the viral diseases that have been studied so far there is a direct correlation between the supplies of nitrogen made available to the host, the severity of symptoms, interference with growth, and multiplication of the virus. There are considerable conflicting data on how the heavyweight virus protein is formed and acquires the ribonucleic acid that gives it virus properties. The plant apparently does not reallocate nitrogenous materials from its tissues to the virus when inorganic nitrogen is in short supply. The nitrogen from the soil is incorporated directly in the precursors of the nucleoprotein so a shortage of nitrogen hampers virus multiplication (Spencer, 1941; Bawden and Kassanis, 1950).

In addition to nitrogen, the supply of phosphorus affects the severity of virus reaction in plants. The tumor tissue of sorrel incited by the clover wound tumor virus has a phosphorus requirement several times as large as that of normal tissues. These effects appear logical since phosphorus is essential for the heavier energy demands of the cell and the formation of nucleotides used in the synthesis of nucleic acids.

This very brief condensation of over 700 original reports may be an oversimplification of the effect of soil fertility. However, it does show that there is a logic to the results being obtained on the effects of host nutrition on plant diseases. When the diseases are grouped according to the physiological basis of their parasitism, the basic effects become apparent.

VII. Host Genetics in Relation to Type of Pathogenicity

The employment of disease-resistant varieties has come to be accepted as one of the cheaper and more effective methods of avoiding the ravages of plant pathogens. The method has become standard for controlling many of the highly specialized parasites such as the rust and powdery mildew fungi. It has been possible to synthesize varieties of cereals, for example, that are highly resistant to specific races of the obligate parasites.

With such records of success before them, enthusiastic novices in plant breeding often assume that all plant diseases should be controlled by use of resistant varieties. They soon discover that resistance to a fruit rot, for example, is harder to achieve than resistance to a rust, and, once resistance is found, it is much more difficult to transfer into a commercial variety by hybridization and backcrossing. Are there any principles that could guide the plant pathologist in making a decision as to whether breeding should be preferred over sanitation, crop escaping, or preventive sprays for disease control? There appear to be such principles because the inheritance of resistance by the host seems to vary with the six degrees of specialization involved in the parasitic processes. This interesting relationship appears worthy of discussion.

1. *Destruction of Food Reserves*

The storage organ rots and decays of the food reserves have rarely been controlled by use of resistant varieties. As emphasized above, these diseases are essentially digestive processes of saprophytes whereby a tremendous reserve of readily available carbohydrates is attacked as soon as the protective covering is injured. About the only chance of securing a more resistant variety is to find one with either a thicker skin or firmer flesh so that it is not wounded so easily. An alternative possibility is to find a variety with a toxin in the tissue that can inhibit the pathogen.

If such sorts of resistance for storage and transit rots are found, it is very likely to impart such undesirable quality to the produce that it will destroy its horticultural value. These attributes, if they did occur and were acceptable, are the product of general growth phenomena that undoubtedly are regulated by scores of genes. The problem of transferring such genes in mass and eliminating all genes that contribute toward susceptibility is a tremendous undertaking. In this type of disease, the advantages rest with the unspecialized parasite that is versatile in its food acceptance and has no special system of establishing itself on an actively growing cell.

2 and 3. *Prevention of Seedling Metabolism and Procurement*

The root rots and seedling diseases are comparable to the storage organ rots in the general omnivorous nature of the parasites, but they differ in that they do attack and destroy living cells. They usually invade through wounds, but they may also form mycelial masses on the plant surfaces and breach the epidermis by pressure or digestive enzymes such as is shown by *Thielaviopsis basicola* on tobacco.

Resistance can be found in mechanical strength of the tissues or the presence of toxic materials such as the phenolic and alkaloid materials in certain crops resistant to Texas root rot (Greathouse and Rigler, 1940). In addition, the plant, even though invaded, may escape destruction by replacing injured tissue. Many maize seedlings have the epicotyl completely destroyed by *Diplodia zeae*, operating from either the seed or the soil; but the timely establishment of a permanent crown of roots from the lower nodes of the stem compensates for the loss of water and nutrients normally provided by the primary root system. "Resistance" of some strains of tobacco to black root rot is almost entirely achieved by prompt development of roots to replace those destroyed by the fungus during periods of weather favorable to the parasitic establishment.

These types of "growth" resistance are regulated by many genes and resistance of this sort is rarely transferred readily from resistant to susceptible lines. There may be two or three major genes for resistance but invariably their effects are modified by a battery of secondary genes, the number of which is so complex that they are rarely determined by the breeder.

4. *Interference with Upward Transport*

The situation changes drastically when one progresses from these relatively nonspecialized soil inhabitors to the more specialized parasites of the transport system. These pathogens have fairly well-defined nutritional requirements which are met best by the environment of the tracheal system. However, in choosing this more selective, specialized environment they automatically have sacrificed some of their freedom of action, i.e., ability to accept wide deviations in the qualitative and quantitative condition of the nutrients in the host. Their fate can be determined by a relatively small change in the nature of the host cell and host metabolism.

It is not surprising, therefore, that most crops attacked by vascular parasites have different degrees of resistance ranging from complete susceptibility through tolerance to near immunity in different varieties. The number of wilt diseases controlled by use of these resistant varieties is legion: sweet corn wilt caused by *Bacterium stewartii;* the wilts of cotton, tomato, peas, bean, cantaloupe, watermelon, pepper, cabbage, etc. caused by *Fusarium* spp.; and even *Graphium ulmi* on elm.

The breeding of this resistance into commercially desirable lines presents no insurmountable problem as a general rule. Usually resistance of this sort is inherited through a relatively few genes (two to six). By reasonable diligence, the resistant hybrid can be backcrossed to the susceptible commercial parent to fix desirable agronomic and horticultural traits, while retaining the major genes for resistance. One advantage is that the wilt parasites apparently are not so prone to develop a multitude of parasitic races as the obligate parasites because they do have an existence apart from their hosts during part of their life cycle.

5. *Destruction of Food Manufacture*

a. *The Leaf Blights, Cankers, and Rots.* The situation in *the leaf blights, cankers, and rots* is highly confusing because of the diversity of the fungi and bacteria involved. Some of them are omnivorous, facultative saprophytes much like the root rot fungi. They attack injured tissues, have no special aptitude either in initiating an attack or in establishing suitable liaison with the functional cells of the host. Among these are many species of *Alternaria*, *Septoria*, *Stemphylium*, *Pseudomonas*, and *Xanthomonas*. Others such as species of *Cercospora*, *Colletotrichum*, and *Venturia* have well-developed parasitic patterns including the production of parasitic races with very specific varietal preference. Breeding for resistance in this group is successful almost directly in proportion to the degree of parasitic specialization exhibited. Breeding is quite rewarding in the latter group but relatively unimportant in the less specialized types.

b. *The Downy Mildews.* The downy mildew fungi, in general, have very well-developed parasitic capabilities ranging from facultative to obligate parasitism. Resistance is relatively easy to develop and transmit by breeding. The outstanding example is the development of varieties of potatoes resistant to *Phytophthora infestans* by transfer of four or possibly five genes from *Solanum demissum* to *S. tuberosum*. The most recently developed resistant varieties constitute about the fifth time that the late blight problem has been "completely solved" by breeding in North America and in Europe. Resistance has been quite ephemeral merely because the fungus has great ability to produce new parasitic races. Currently there is evidence that resistance being developed in Mexico may be more enduring (Niederhauser, 1956). Resistance to this general class of parasites seems to be regulated by a relatively few major genes, since only one to three are required for resistance to a single biotype of the fungus.

c. and d. *The Powdery Mildews and Rusts. The obligate parasites* of the Erysiphaceae and Uredinales have highly specialized parasitic races. The physiological relationship of host and parasite is so delicately balanced that a small change in the host protoplast may be detrimental to the parasite. Although very little is known about the physiology of the obligate parasite or the specific requirements made upon the host, it is obvious that in establishing a partially commensal state with the host, these fungi have sacrificed most of their independence of action. The obligate parasites are not versatile enough to accept the biochemical changes induced in the host by two or three new genes. Resistance to a single biotype usually is regulated by a single gene or pair of genes. Because of the prevalence of monohybric and dihybric inheritance of resistance, breeding of new varieties is a relatively simple matter. It is equally simple for a new race of the parasite to circumvent such highly specialized forms of resistance by reorganization of its own genes.

Because of this interplay of genes of the host for resistance and those of the parasite for invasiveness, Flor (1946) has suggested that there are matching genes in *Melampsora lini* and its host. This intriguing concept must be true at certain levels but there are undoubtedly many genes that operate independently because a rust and its host do have many functions that are independent of each other. Only time and the acquisition of knowledge on the biochemistry of the host-parasite relationship in obligate parasitism will reveal to what extent there are matching of genes for resistance in the host and for parasitism in the pathogen.

6. *Diversion of Foodstuff to Abnormal Uses*

The Galls. It should be pointed out that certain types of fungi, still classified as obligate parasites, have rather wide host ranges and are not readily controlled by breeding for resistance. Among these are certain members of the lower fungi such as *Plasmodiophora brassicae,* the cause of clubroot. Many efforts to develop resistance in its various cruciferous hosts met with uniform failure until within the past 20 years (Walker, 1936). This experience shows that parasites which are not readily cultured in artificial media do not necessarily have to be highly specialized in their host preference. Their major nutritional requirements may be met by some common function of photosynthesis or protein metabolism but apparently they are not so highly specialized that they may be destroyed by relatively minor changes in the host.

In general, there have been variable degrees of success in breeding for resistance to those pathogens that operate as growth regulators. Probably this is attributable to the fact that some of them are relatively poor parasites. Some are unspecialized facultative saprophytes that have an unusual ability to produce extracellular chemicals that have plant growth stimulating ability while others are obligate parasites.

Many of the pathogens such as viruses that regulate cell functions from within the tissues without causing necrosis are highly specialized, and almost without exception can be controlled by certain forms of resistance. For example, the parasitism of cereal smuts which converts ovary tissue into an overexpanded mass of mycelium and host cells apparently is controlled by two or three genes in many varieties. Likewise, there is ample evidence that the susceptibility to viruses may be changed by replacing one to three genes.

VIII. Search for Effective Disease Control by Application of Logical Principles

Plant pathology has one ulterior purpose—the prevention of plant disease. Therefore, much of the knowledge and skill of the profession must be directed toward *control of the forces of pathogenesis.* Any idea for controlling pathogenesis should take into account that disease is just as much the product of host reaction as it is the result of a parasitic invasion.

A. *Methods of Regulating Pathogenesis*

Much more is involved in this concept of disease control than the ordinary idea of destroying or avoiding pathogens. It is based upon the obvious truth that a parasite may be tolerated if means can be developed to regulate or circumvent its ability to induce disease. As mentioned in the opening paragraphs and as illustrated by several examples in subsequent discussions, parasitism may play a minor role in many diseases.

This idea of regulating the forces of pathogenesis as well as exterminating the pathogen is entirely sound because the records show that man has never learned "to control" any of the major pathogens. About all that has been achieved is to find methods of living with them—but not at their mercy. How many have ever been eliminated (i.e., eradicated entirely) by breeding, application of protective chemicals, sanitation, disinfection of the environment, etc.? Not one! The nearest approaches have been in the breeding of the Washington varieties of asparagus for resistance to *Puccinia asparagi*, an autoecious rust, and in eradicating citrus canker (*Xanthomonas citri*) in Florida by burning all infected groves. But the rust can still be collected today on wild asparagus and citrus canker does flare up at times so these are horticulturally successful measures rather than absolute victories over the pathogen.

A tolerable situation such as this can be established by: (a) use of disease-escaping tactics, (b) suppression of the pathogens to permissible levels, or (c) use of disease-resistant or tolerant varieties of crops to establish a favorable host-parasite balance. Many prevalent diseases can be escaped by use of disease-free seed, selection of sites unfavorable to the pathogen, choice of favorable planting dates, avoiding wounds and bruises, use of quarantines, etc. The prevalence of the pathogen can be suppressed by seed treatment, soil disinfection, protective foliage sprays, space fumigation of soil and storage areas, stimulation of antibiosis, or rotation of crops. The disease response of plants to infection can be ameliorated by antidoting toxins, proper fertilization of soil, encouragement of new root growth, avoiding extreme fluctuations in soil moisture and available nutrients, etc. Resistant crops can be developed by selection, hybridization, and grafting on resistant root stocks.

Which one of these various methods is likely to be most successful against a specific pathogen? Does the physiological basis of organizing the knowledge on plant disease offer any help in developing some sound principles? If the principle is fundamentally sound, it should be useful in directing research activities into fruitful channels. In order to draw the facts together in an intelligible manner it is well to study the interrelationship of various factors in an epidemic.

B. *The Factors in an Epidemic*

Any disease outbreak depends upon three elements: the inherent susceptibility of the host, the inoculum potential of the parasite, and the impact of environment on parasitism and pathogenesis. Each of these has two components as indicated schematically by the triangle of epiphytosis in Fig. 1. The height of the apex (severity of the epidemic) is determined by the relation of these six components.

The base for any epidemic is the susceptibility of the host. This susceptibility is composed of two factors: its seasonal development which exposes it to the pathogen, and its inherent resistance to invasion or tolerance of the forces of pathogenesis. If either of these elements is shortened as indicated in Fig. 1 from D-10 to D-2 on one hand or from I-10 to I-2 on the other, the host will either escape the infection periods or resist entering into a pathogenic process. Even if all other elements remain the same, damage to the crop could be reduced from 100 units to 20 or less as the base of an epidemic is reduced. If either side of the base is reduced to 0, obviously there can be no crop damage.

Hinged to the seasonal development of the crop is the influence of environment. If the host develops at the proper season, it may encounter conditions that will be favorable to the pathogen and to its own disease reaction. If the various climatic and edaphic factors of temperature, moisture, acidity, etc., are properly balanced, the environment becomes more conducive to disease development. These could be indicated by the length of the left-hand side of the triangle. However, the full impact of environment depends on the frequency and duration of these periods which are usually designated as infection periods. If the periods are reduced to zero, there can be no disease so this force operates as the angle on the left-hand side of the triangle to determine whether maximum damage will be 100, 50, or 0 units, for example.

The virulence and, to a large measure, the prevalence of the parasite will be determined by the inherent susceptibility of the host as described in the section on host-parasite balance. The virulence of the pathogen, unfortunately, has never been fully divorced from the innate properties of the host so no one has been able to reduce inherent virulence for a given host in a practical way. Even if methods for causing dissociation of avirulent strains were developed, the forces of selective evolution would prevent survival of such strains under field conditions.

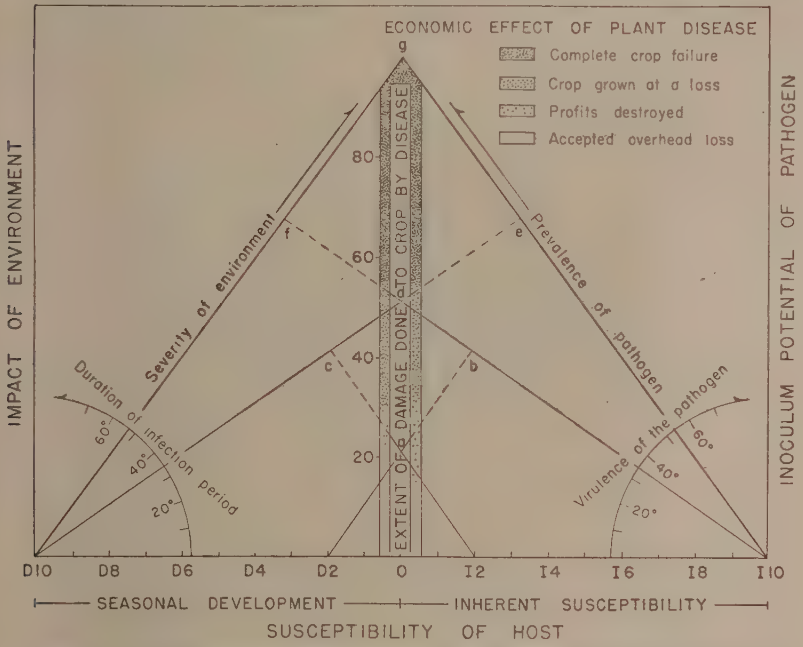
ECONOMIC EFFECT OF PLANT DISEASE
g
▨ Complete crop failure
▨ Crop grown at a loss
▨ Profits destroyed
☐ Accepted overhead loss
EXTENT OF DAMAGE DONE TO CROP BY DISEASE
80
60
40
20
f
e
c
b
Severity of environment
Prevalence of pathogen
Duration of infection period
Virulence of the pathogen
60°
40°
20°
60°
40°
20°
IMPACT OF ENVIRONMENT
INOCULUM POTENTIAL OF PATHOGEN
D10
D8
D6
D4
D2
0
I2
I4
I6
I8
I10
SEASONAL DEVELOPMENT
INHERENT SUSCEPTIBILITY
SUSCEPTIBILITY OF HOST

FIG. 1. The triangle of factors that limit an epidemic. The full impact of a pathogen may be avoided either by reducing host susceptibility, inoculum potential of the parasite, or environmental conditions favorable to pathogenesis. The peak may be reduced from a maximum point of "g" to some lower level such as a, b, c, e, or f by restricting one or more of the six factors in an epidemic, as indicated by the internal lines. This would reduce crop losses from a maximum of 100 to some lower figure such as 50, 20, or even 0. All figures are assigned empirically and cannot be given precise values at this stage of knowledge. The preferred treatment for a disease shifts progressively clockwise from the left corner to the lower right side of the base as the specialization in pathogenesis increases from class 1 to class 6 of disease.

Only one basic rule seems to emerge. As the inherent susceptibility of the host is reduced by breeding or selection, the angle of virulence increases so the parasite tends to maintain a constant inoculum potential. Disease control must depend on reducing the prevalence of the parasite (shortening the right side of the triangle) or disrupting the degrees of virulence by frequently changing the genetics of the host. Schematically, the severity of an epidemic could be held at 50 units either by reducing the prevalence of the pathogen to "e" or the angle of virulence to 35 degrees, as shown in Fig. 1.

C. *Relation of Class of Parasitism to Disease Control*

Disease control, therefore, may be achieved by disrupting any one of six factors in the epidemic triangle. Which one of these factors is most likely to be successful? It depends strictly upon the type of pathogenesis involved. For example, if all the disease prevention methods currently used were enumerated for diseases representative of each class of pathogens, they could be classified somewhat as indicated in Table I.

The more poorly specialized parasites of the storage organs (class 1), which are nothing much more than opportunistic facultative saprophytes, are controlled by use of disease-escaping tactics—avoid wounds, harvest fruit when mature, dry immediately, prevent sweating in storage, etc. In short, disease controls are applied at the left-hand base and angle of the epidemic triangle. The second alternative is to regulate the environment as much as possible, and the third measure is to reduce the population of parasites. Scarcely a thought is given to inherent resistance. In other words, there is diminishing likelihood of success in developing preventive methods as one progresses clockwise around the triangle from the left angle.

The seedling diseases (class 2) which are so analogous to the rots in many respects have only slightly less possibility of control by disease-escaping measures; but there is much more emphasis on changing the environment. Seed are sown in well-prepared seed beds of proper soil texture and are treated with chemicals or the soil is treated. In other words, the emphasis in disease control shifts one more step clockwise on the triangle but does not reach over to inherent resistance.

Control of the root rots (class 3), which are one stage more highly specialized as parasitic pathogens, depends very heavily on proper choice of environment but, by far, the major emphasis is on reducing the prevalence of the parasite by various soil treatments. A form of disease resistance, albeit very imperfect, may be developed for a few of the root rots. Usually, this is a genetically controlled growth response in which the host recovers from infection although this is not a positive

TABLE I
SUMMARY OF DIFFERENT TYPES OF CONTROL MEASURES FOUND TO BE EFFECTIVE FOR DIFFERENT CLASSES OF DISEASE

Class number and type of disease	Effective class of control measures		
	Preferred method	Second choice	Third choice
(1) Rots of storage organs	Avoid wounds	Improve storage	Chemical treatment
(2) Seedling diseases	Chemical treatments	Better culture	Choice of sites
(3) Root rot diseases	Crop rotation	Stimulate antibiosis	Treat soil, resistance
(4) Vascular diseases			
(4a) Soil	Resistant varieties	Choice of site	Crop rotation
(4b) Insect	Resistant varieties	Insect control	Crop rotation
(5) Leaf diseases			
(5a) Leaf blights and cankers	Chemical protection	Resistant varieties	Sanitation
(5b) Downy mildews	Chemical protection	Resistant varieties	Sanitation
(5c) Powdery mildews	Resistant varieties	Chemical protection	Sanitation
(5d) Rusts (autoecious)	Resistant varieties	Crop rotation	Chemical protection
(5e) Rusts (heteroecious)	Resistant varieties	Eradicate Alt. host	Chemical protection
(6) Utilization diseases			
(6a) Floral smut	Resistant varieties	Seed treatment	Sanitation
(6b) Root galls	Crop rotation, soil treatment	Sanitation	Avoid wounds, resistance
(6c) Viruses (mosaic)	Resistant varieties	Seed certification	Sanitation
(6c) Viruses (yellows)	Resistant varieties	Sanitation	Vector control

form of immunity. In other words, the root rots, most of which are facultative parasites, are controlled by changing the factors one more step clockwise on the chart than for the seedling diseases.

While discussing the soil microorganisms, it might be well to consider the more highly specialized gall forming (class 6) parasites that are capable of invading roots and regulating their growth (6b). Nearly all of these have an existence apart from the host so special effort is made to reduce their population in soil. However, there may be definite forms of resistance in varieties, so breeding may be more valuable than for any of the other diseases discussed above. Control measures rotate clockwise to the right-hand side of the triangle.

The wilt diseases (class 4) further demonstrate that type of control measure is largely determined by the physiological specialization in the parasite. Disease resistance becomes very important with secondary consideration to methods of avoiding the pathogen or reducing its prevalence. Crop rotation is important and avoidance of vectors is a major consideration.

The same series of changes may be observed in disease-prevention measures for the pathogens of aerial organs (class 5). In the least specialized leaf blights, efforts are made to avoid the pathogen by choice of sites and planting dates, but most of the control revolves around use of chemicals to reduce the prevalence of the parasite. A great many of the leaf blights and downy mildews are sufficiently specialized so they can be avoided by use of disease-resistant or tolerant varieties. The emphasis upon disease-resistant or even immune varieties increases as obligate parasites are examined. The more highly specialized pathogens of the utilization system of the plant (class 6) such as the smuts, the powdery mildews, rusts, and viruses are very susceptible to hereditary changes in the physiology of the host.

Although there are minor exceptions to the sweeping generalities made here, it is interesting that a classification of disease based on the physiology of pathogenesis leads to such an orderly understanding of disease-control measures. This is not a surprising revelation because the system aligns the parasites in a more or less orderly fashion according to the specialization in their parasitic abilities.

The pathogen that is divorced from its susceptible host most of the time is very susceptible to changes in the environment. Those that destroy the host most readily when it is handicapped by unfavorable environment are less destructive when conditions are made favorable to the host's growth. The parasite that is exposed on a leaf, even for a brief period in moving from leaf or twig to new susceptible tissue, can be suppressed by chemicals; but if it is so highly specialized that it has no existence apart from its host, the metabolism of the host cell is the dominant feature in determining how successful its parasitism will be. Such parasites are most readily suppressed by use of resistant varieties.

IX. Discussion and Summary

When the efficiency of a plant is so reduced that it cannot make maximum use of the factors of its environment for growth and reproduction, that plant must be considered as diseased. Efficiency may be impaired by disrupting any one or more of the several basic physiological processes involved in the synthesis and utilization of foodstuffs. Therefore, plant disease is essentially a physiological process of the plant regardless of whether it is incited by physiogenic or pathogenic agents in the environment.

Most plant diseases are caused by pathogens, so pathogenesis is of major concern. Pathogenesis is the result, in many but not all diseases, of a parasitic establishment. The direct effects of parasitism, however, may be rather mild even though the loss of food materials does constitute a drain on the host's efficiency. However, the further exacerbation of disease condition is more often due to the foreign chemicals produced either by the pathogen or the invaded tissues. Extracellular materials such as the enzymes that dissolve cell walls, the toxins that poison metabolic processes of the cell, or the growth regulants that influence the rate of cell multiplication and differentiation are vitally important in determining pathogenesis in plants.

The postulate is advanced that parasites are in a constant process of evolution in which they progress from nonparasitic associative relations with the host through facultative saprophytism and parasitism to obligate parasitism. Simultaneously, there is a reduction in the tendency to destroy host cells by secreting digestive enzymes and the pathogen tends to become associated with the host cell in a compatible arrangement. The habitat of the parasite in the host changes from a general cell invasion to a more specific intercellular establishment. The ultimate in parasitism is found as an intercellular invasion with haustoria in the cell or even an external habit with haustoria in the host cell as in the Erysiphaceae.

Concurrently with these changes, the host becomes more responsive or even actively reactive to invasion so that in the more advanced types of parasitism there is an essentially commensal state existing between host and parasite. The parasite curbs its destructiveness (pathogenicity) and seeks out a compatible intracellular association where it can procure food or regulate cell activities from a point of contact near to the host nucleus. In the most advanced stages of evolution the parasite regulates activities of the host cell for its own gain, and may even be commensal with it.

There is substantial evidence in support of this hypothesis that parasitism evolves toward commensalism by increased specialization and skillful change in the type of disease-inducing chemicals released. The host and parasite come into a balanced state either by physiological adjustment on the part of the parasite or by selective hereditary changes in the host. An understanding of this process of evolution in parasitism seems to be a prerequisite to formulation of programs for disease control.

The specialization of the parasite apparently depends upon the intricacy of the host function that it affects. The destruction of a food reserve or digestion of a dead cellulose cell in wood or fiber can be achieved by any saprophyte with the proper enzymes provided it has tolerance for the cell environment. The general destruction of nutritious cortical cells in the seedling tissue or in roots, so as to prevent establishment of water-procuring organs, is only one stage more specialized. However, selective invasion of special cells such as the tracheal tubes and use of the nutrients of the tracheal sap necessitate much more specialization. The parasite sacrifices a measure of its independence and becomes more dependent upon the host's physiology. With only minor variations this specialization becomes intensified as the pathogens become more adept at operating on or in the cells that are actively photosynthesizing foodstuffs where they cause various forms of leaf blight. Any interference in the transport and assimilation of foods into new cells and tissues without outright destruction of the tissues constitutes a very delicate adjustment, particularly when the metabolism of the cell is altered or growth processes are changed as occurs in various regulatory diseases such as viruses.

When diseases are classified into six groups according to this scheme, without concern for the taxonomic classification of the parasite or the particular hosts involved, the orderly arrangement proves very valuable in understanding the nature of disease. Examples were offered to show how this order can be used to explain much of the influence of environmental conditions on disease, why host nutrition affects various diseases differently, how host genetics influence parasitism, and what sort of disease-control practices are preferred for different diseases.

It is only natural that this system of organizing the knowledge on plant diseases would cast new light on such considerations because the system is based on physiological considerations that are basic to parasitism and pathogenesis. Because this approach reaches beyond superficial trivialities and provides basic principles, it should be valuable in organizing research and in teaching the principles of plant pathology. It is easier to remember details and judge circumstances when they are coordinated in a logical scheme rather than as isolated events.

Only the most primitive effort can be made today to classify diseases as physiological functions of parasitism because so little is known about the biochemistry of disease processes (pathogenesis). This is to be regretted very deeply because it prevents maximum progress in solving disease problems in plants. Probably an equally great disservice is being performed for other professions because knowledge of the basis of pathogenicity could cast much light upon physiology of plants in general.

Indicative of the great contributions that plant pathologists could provide the world by intensive research on the physiology of pathogenesis are such achievements of the past as the studies on the pectinase enzymes in vegetable soft rots by Jones (1909), the development of new classes of plant growth regulants in the gibberellins isolated from the bakane disease of rice by Kurosawa (Stowe and Yamaki, 1957), the new concept of an autocatalytic growth incitant in crown gall by Braun and Mandle (1948), and the discovery of a new amino acid (tabtoxinine) in the toxin from wildfire of tobacco by Woolley *et al.* (1952b).

Such knowledge has significance far beyond the boundaries of plant pathology. Hundreds and possibly thousands of such secrets are hidden in the galls, the blights, the rots, the pigment changes, the abscission of leaves, and many other symptoms. These mechanisms of pathogenesis could be the key to an understanding of cancer or could cast new light on the forces that make plant cells grow and differentiate into new tissues.

Never was the time better for launching investigations of this sort. The chemists and physicists have placed at the disposal of biologists new techniques and analytical tools that are most expedient and reliable. There is no longer any sound excuse for not investigating the amino acids, the organic acids, the keto acids, the sugars, the growth regulants, the pigments, the toxins, or any other class of chemicals wherever the imagination would lead an investigator.

The tools of paper chromatography, column chromatography, electrophoresis, paper ionophoresis, ultraviolet and infrared spectrophotometry, and many others await full use in this field. They are the means of gaining a full understanding of the forces behind pathogenesis. With understanding, far better than that proposed above, will come progress in altering the metabolism of the host so as to incite acquired resistance to parasites, in developing new chemicals to inhibit pathogens or alleviate their damage, and in improving the processes of breeding for inherited resistance.

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CHAPTER 3

The Multiplication of Viruses

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I. DIFFERENCES BETWEEN VIRUSES AND ORGANISMS

Perhaps the best way to introduce our subject is to devote a few sentences to considering why the multiplication of viruses needs to be described and discussed separately from the multiplication of pathogenic fungi and bacteria. The reasons are simple but fundamental; they do not lie in the fact that different techniques need to be used to study viruses, but in the fact that viruses are structurally and metabolically different from even the smallest microorganism. At least this is true of all the viruses about whose constitution anything precise is known and our discussion will mainly be restricted to these. Because of this restriction it is advisable at the start to interpolate a cautionary note about the dangers of generalization. The viruses whose chemical nature has been determined cover a variety of types differing considerably in host range, stability, and methods of transmission, and the fact that they have all proved so similar certainly means that many others will also resemble them, but it is still premature to assume that they are representative of the whole group, and there may be other types, some perhaps more nearly resembling organisms.

For the first 40 or so years after the existence of viruses was first recognized, there were only two features that separated them from pathogenic organisms. One was their invisibility under high powers of light microscopes and the other their apparent inability to multiply on artificial media. Neither necessitated the conclusion that virus multiplication must be a different process from the growth and fission of cells, for there were many unequivocal organisms that seemed to be obligate parasites and there were also saprophytes not much larger than the minimum size that is clearly resolved by microscopes using visible light. Against these two essentially negative features could be placed the positive similarities with organisms, the indisputable facts that viruses multiply readily in susceptible living cells, and that, although like usually begot like, multiplication occasionally led to offspring differing in pathogenic properties from its parent. Ability to multiply and vary are generally accepted as prime characters of living systems, so it is hardly surprising that, despite a few dissentient voices, the most generally held view was that viruses were, in essence, ultramicroscopic organisms and that they probably multiplied by a process similar to binary fission.

In the 1930's, however, information about the chemical constitution and structure of viruses began to accrue as, increasingly, the methods that previously had proved successful in the isolation and study of enzymes were applied to extracts from virus-infected plants. This information made it almost immediately apparent that analogies with bacteria were wholly inapt and that viruses represented an altogether different kind of biological category. The first advance, as often happens, came from work with tobacco mosaic virus, when it was shown that infected plants contained large quantities of a specific nucleoprotein, preparations of which were infective at high dilutions. That tobacco mosaic virus was not exceptional was soon demonstrated, for plants infected separately with one of several other viruses were also shown to contain specific and characteristic nucleoproteins. There is no call here to review the great amount of work then done to identify these nucleoproteins with the viruses themselves; it will suffice to say that results from many kinds of experiments related the two and that, although a carping critic could justifiably say that the evidence of identity still falls short of proof, it can be categorically stated that, if these nucleoproteins are not the viruses themselves, then the viruses must be even simpler entities and be even less similar to organisms. I shall take the now generally accepted view, and the one favored by all the evidence, that these nucleoproteins are the viruses.

When purified preparations of plant viruses were first obtained, the ability of some to form crystals or liquid crystals seemed to arouse more interest than did their chemical nature. The main biological significance of crystal formation was, however, simply to indicate a uniformity of particle size that would be unusual with organisms and that seems incompatible with multiplication by binary fission, that is by particles growing and then dividing into two. The main value of crystallization, apart from its use to fractionate preparations, was that it allowed the techniques of X-ray diffraction to be applied to the study of viruses. These techniques not only provided the first accurate and direct measurements on the size of virus particles, but, more important, gave information on their internal structure. They showed that the particles were composed of similar units, repeating in space with a three-dimensional regularity, very different from the continuously varying structure inside a living cell. Indeed, it was then clear that, in their chemical simplicity, consisting as they do of only two components, nucleic acid and protein, and in their fixed pattern of structure, these virus particles were more nearly analogous to single components of cells than to the complex organization characteristic of whole cells. Further work has strengthened this conclusion and has failed to reveal any enzymes or metabolic activities intrinsic to the virus particles. As they are formed only in environments in which other nucleoproteins can be synthesized, they seem to depend on their hosts, not simply for a supply of the materials from which they are made, but also largely for the systems that make these materials.

The old concept of a virus disease as the result of one organism preying on another cannot be fitted to modern knowledge; instead, it must be replaced by the idea of virus multiplication as a derangement of the nucleoprotein metabolism of the host, with infection changing the cell's metabolism and leading to different end products of its synthesis. The viruses are not simply parasites drawing food from their hosts; the relationship between the two is much more intimate than that and, in effect, the viruses become determinant parts of the host cell and their multiplication is one sequel of the metabolism of the whole cell.

In uninfected cells, protein synthesis is closely correlated with and dependent on the synthesis of nucleic acid, and it is the intrusion of the virus nucleic acid into host cells that seems to be the prime factor in disturbing their metabolism. For some 20 years after viruses were first identified as nucleoproteins, these characteristic nucleoprotein particles seemed to be self-replicating and to be the minimum requirements for initiating infection. Recent work with tobacco mosaic virus, however, makes this idea untenable, for it has shown that preparations free from any of the characteristic rod-shaped particles of nucleoprotein, and consisting largely and possibly exclusively of nucleic acid, can be infective. Hence, the protein as such does not multiply, whereas the nucleic acid seems not only to reproduce itself, but also to be responsible for synthesizing the characteristic protein that normally accompanies it in the complete virus particles.

II. The Constitution and Structure of Viruses

Direct observations on the mechanisms involved in virus multiplication are made impossible by the facts that viruses are not resolved by microscopes that use visible light and that they multiply only intracellularly. That they can be resolved by electron microscopes is helpful to only a limited extent because the insides of living cells are not susceptible to exploration by electron microscopy. Improvements in the techniques of preparing ultrathin sections of cells may ultimately allow electron microscopy to provide unequivocal evidence about the sequence of events that is initiated by infection, but at present these can only be inferred from indirect evidence. Any discussion of the means whereby viruses multiply, therefore, must of necessity be speculative, based on a knowledge of their constitution and structure, of the factors that affect multiplication and of the specific products that can be identified in infected cells. For some kinds of work, bacterial viruses are much more amenable to study than are viruses that infect higher plants, and for even a rough outline sketch of the multiplication of viruses in higher plants we must rely greatly on analogies with bacterial viruses. First, however, let us consider what is known about the constitution and structure of viruses, as a necessary background to understanding what virus multiplication necessitates.

Of the dozen or so plant viruses that have been purified to the state in which chemical analyses have any significance all have been found to be chemically similar, and to contain only protein and ribonucleic acid. Some of these have spherical, or near spherical, particles, others are elongated, some seemingly rigid rods, and some flexible filaments. The ratio of nucleic acid to protein is constant for each virus, but the proportion of nucleic acid differs in different viruses. In those with elongated particles, such as tobacco mosaic and potato X and Y, the nucleic acid amounts to only $\sim 5\%$ of the weight of the particles; in the spherical viruses, it is considerably more, and ranges from 15% in alfalfa mosaic, to 18% in tomato bushy stunt and tobacco necrosis, 21% in southern bean mosaic, 35% in turnip yellow mosaic and 40% in tobacco ring spot virus (Knight, 1954).

All viruses yet analyzed from flowering plants contain ribonucleic acid, but this is not true of all viruses. All the bacterial viruses examined contain deoxyribonucleic acid, as also do many viruses that cause polyhedral diseases of various species of insects; these all have considerably larger particles than the plant viruses known to contain ribonucleic acid, and in this respect they resemble some other plant viruses such as potato yellow dwarf and wound tumor, which multiply not only in plants but also in the insect vectors (leaf hoppers) that spread them from plant to plant (Black, 1955). Unfortunately, these large plant viruses are unstable and do not occur in either plant or insect host in high concentration, and their constitution is unknown. I mention them here to stress again the fact that the kinds of viruses with which I shall later be concerned may not be representative of the whole group; there is the obvious possibility that these large ones may differ chemically from the small viruses, and even if not more complex in the sense of containing more than two components, they may perhaps contain deoxyribonucleic acid instead of ribonucleic acid. The main chemical differences between the two kinds, lie in the identity of the sugar, ribose in one and deoxyribose in the other, and that the pyrimidine uracil in ribonucleic acid is replaced by thymine in deoxyribonucleic acid.

A. *Chemical Analysis*

There are various ways in which the protein and nucleic acid of the small plant viruses can be separated, and detailed analyses have been made on the two components from some. The contents of various amino acids in the protein moiety from several strains of tobacco mosaic virus have been determined, and it is clear that those that are similar in their physical properties and are closely related serologically also have very similar amino acid constitutions. Strains that differ in physical properties and have many distinctive antigenic groups, however, also contain different quantities of individual amino acids and some strains contain an amino acid that does not occur in others (Knight, 1954). This does not mean that single genetic changes are immediately reflected in a quantitative change in the amino acid composition of the protein; indeed, the kinds of change most often reported, such as from type tobacco mosaic virus to the masked strain, or from yellow to green strains of tomato aucuba mosaic virus, do not measurably affect the amino acid composition (Table I).

Differences have been detected mainly between naturally occurring strains of the virus whose origins are obscure, but there are also examples of what seem to be mutant forms differing in their amino acid constitution from their parent types; for example, strain U2 differs from type TMV (Siegel and Wildman, 1954) and the bean form of cowpea mosaic virus contains histidine, which does not occur in the tobacco form (Bawden, 1958). Tomato bushy stunt virus has an amino acid composition very different from that of tobacco mosaic virus, and various strains of it did not differ detectably (De Fremery and Knight, 1955).

TABLE I
THE AMINO ACID CONTENT OF STRAINS OF TOBACCO MOSAIC AND TOMATO BUSHY STUNT VIRUSES[a]

	Tobacco mosaic strains						Tomato bushy stunt strains		
Amino acid	T	M	GA	YA	R	CV4	3	9	10
Alanine	4.1	4.2	4.1	4.1	5.1	4.9	4.7	4.9	4.8
Arginine	8.8	8.9	10.0	10.0	10.0	8.3	5.7	5.9	5.7
Aspartic acid	11.7	11.7	11.7	11.7	10.9	11.3	9.2	9.5	9.2
Cysteine	0.6	0.6	0.6	0.6	0.6	0.0	0.6	0.6	0.6
Glutamic acid	9.9	10.0	10.0	10.0	13.6	5.7	5.0	5.1	4.9
Glycine	1.4	1.4	1.5	1.5	1.0	1.1	4.0	4.2	4.1
Histidine	0.0	0.0	0.0	0.0	0.6	0.0	1.2	1.3	1.2
Isoleucine	5.7	5.7	4.9	4.9	5.1	4.0	2.7	3.0	2.7
Leucine	8.0	8.0	8.0	8.0	8.0	8.1	8.9	9.1	8.9
Lysine	1.3	1.3	1.3	1.3	1.3	2.1	3.1	3.1	3.1
Methionine	0.0	0.0	0.0	0.0	1.9	0.0	0.7	0.9	1.0
Phenylalanine	7.5	7.5	7.5	7.5	4.8	8.7	3.7	3.9	3.7
Proline	4.9	4.9	4.9	4.9	4.9	4.8	2.8	2.7	2.7
Serine	6.0	6.0	6.0	6.0	4.7	7.8	5.6	5.3	5.5
Threonine	8.4	8.4	8.4	8.4	7.0	5.9	8.1	8.2	8.0
Tryptophan	1.9	1.9	1.9	1.9	1.3	0.5	0.6	0.6	0.6
Tyrosine	3.4	3.4	3.4	3.4	6.1	3.3	3.1	3.4	3.3
Valine	7.8	7.8	7.8	7.8	5.3	7.5	7.3	7.0	7.2

[a] The results are from Knight (1954) and De Fremery and Knight (1955) and are expressed in terms of gm. of amino acid residue per 100 gm. of virus. Strains of tobacco mosaic virus are T = type; M = masked; GA and YA = green and yellow tomato aucuba; R = ribgrass; CV4 = cucumber virus 4.

The molar proportions of the bases adenine, guanine, cytosine, and uracil contained in the nucleic acids of five viruses are given in Fig. 1 (Markham, 1953), which shows that each virus contains the four in different proportions. By contrast, when related strains of one virus have been analyzed, no differences have been detected in the proportions in which the different bases occur. The one exception to this statement is cucumber virus 3, which is remotely related to tobacco mosaic virus, but contains more uracil and less adenine (Knight, 1954). The characteristic properties of different types of viruses, then, seem to be correlated with a nucleic acid containing characteristic amounts of the purine and pyrimidine bases, but there is no evidence that variations within types depend on quantitative changes in the components of the nucleic acid. Hence, there can be large quantitative differences in the amino acid constitution that are not reflected in comparable changes in the purines and pyrimidines.

As the identity of the protein is determined by the nucleic acid, clearly this must differ in different strains and change when a virus "mutates" in the course of multiplying. Quantitative changes in the amounts of individual purines or pyrimidines too small to be detected by current analyses could be responsible, but there is no need to suspect anything other than qualitative differences. Analysis of the relative proportions of the four nucleotides gives only a part of the information about the structure of the nucleic acids, and biological specificity is as likely to depend on the order in which the nucleotides are arranged as on their relative proportions. And, with four different kinds of nucleotides, and a total of a thousand or so in the nucleic acid contained in one virus particle, there are more than ample opportunities for different spatial arrangements.

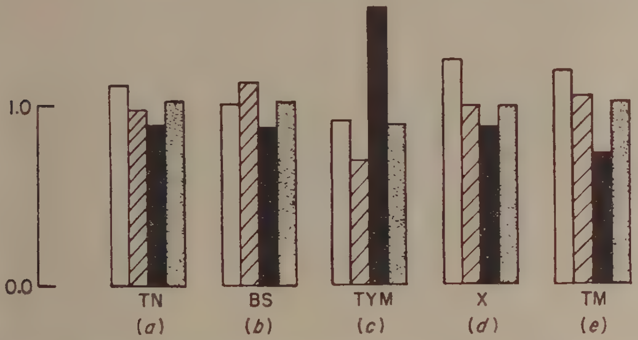

FIG. 1. The molar proportions of the bases adenine (open), guanine (diagonal shading), cytosine (solid), and uracil (dots) in ribonucleic acids from five plant viruses: (a) tobacco necrosis (bean stipple streak), (b) tomato bushy stunt, (c) turnip yellow mosaic, (d) an average potato virus X, (e) a representative tobacco mosaic (tomato mosaic).

Perhaps the conclusion from the detailed analyses of the protein and nucleic acid that is most relevant to virus multiplication is that the plant viruses seem to be made from the same kinds of fundamental units, the same amino acids and nucleotides, as make up "normal" proteins and nucleic acids. As they contain no bizarre substances, there is no call for infection to create any new synthetic systems in the host cells, but only to divert into virus particles substances the cells already make and that otherwise could have formed host components. The lack of any unique components again indicates the importance of structural arrangements in determining biological characters, for viruses act as viruses, not simply because of what they contain, but because of the way in which their contents are put together. As bricks, mortar, and windows might be used to make a house, factory, or church, so amino acids and nucleotides can be arranged in different ways to give end products of different sizes and with different functions.

Again, though, a word of caution is needed against generalization. The fact that tobacco mosaic virus contains no unusual components does not mean that all plant viruses are free from them; already it is known that the nucleic acid of the bacteriophages T2, T4, and T6 of *Escherichia coli* contain, instead of the cytosine usual to deoxyribonucleic acid, the base 5-hydroxymethylcytosine, which has not been observed in any other virus, or in the nucleic acids from any cell, including the host bacterium (Cohen, 1955). Hence, unusual bases do occur in some virus nucleic acids, and viruses that contain them stimulate the synthesis of these bases in cells where they normally do not occur. As only a small minority of viruses has yet been examined, it is unlikely that all those with unusual components have already been discovered, and there seems no reason why some plant viruses should not fall into this category. However, the constitution of tobacco mosaic virus makes it clear that the ability to infect and reproduce, in other words to act like a virus, does not necessitate possessing any novel constituents, and further evidence for this conclusion is provided by bacteriophages T3 and T5 of *E. coli*, which seem to contain none, and their nucleic acid has the usual cytosine.

B. *The Size and Arrangement of Subunits*

The amino acid analyses of tobacco mosaic viruses also provide methods of calculating the size of subunits from which the protein is built. The enzyme carboxypeptidase, which hydrolyzes the C-terminal residues of peptide chains, releases threonine from all strains of tobacco mosaic virus tested, without affecting infectivity (Harris and Knight, 1955). If each threonine residue released is assumed to represent one peptide chain and the mean weight of virus particles is taken as equivalent to a molecular weight of 50×10^6, the subunits would have a molecular weight equivalent to about 17,000. Similarly, from the content of cysteine, the amino acid present in least amount, a subunit of 18,000 can be calculated on the assumption of one cysteine molecule per subunit (Knight, 1954).

Information on the way in which the subunits are put together comes from X-ray diffraction studies. These show, and there is supporting evidence from electron microscopy, that the seemingly rigid rod of tobacco mosaic virus is a hollow tube of maximum radius of about 9 $m\mu$, and that the subunits are set in a spiral about the long axis of the particle, with, probably, 49 units in three turns of the spiral. The tube seems to be lined with protein, and the nucleic acid has its phosphate-sugar chain at a radial distance of 4 $m\mu$, where it is deeply embedded in protein, with its chain direction related to the spiral arrangement of the protein units (Franklin *et al.*, 1957). The position of the nucleic acid seems established, not only by measurements on intact virus particles, but by comparisons of these with morphologically similar rods that can be made by reaggregating disaggregated protein. When the virus is treated with alkali at pH 10.5, the protein breaks down into fragments with average molecular weight of about 100,000, probably six of the subunits; the nucleic acid separates from the protein and it can be removed by electrophoresis. When the protein preparation is acidified, rods form that in the electron microscope resemble normal virus particles (Schramm, 1947), but these have a density minimum at a radial distance of 4 $m\mu$, instead of the density maximum found by X-rays in the nucleoprotein particles.

That the nucleic acid is carried toward the center of the particle of tobacco mosaic virus is also suggested by examining disrupted particles with the electron microscope. In alkali-treated preparations from which the nucleic acid has been removed, fragments occur of the general size and shape of the cross-section of virus particles but with central holes, presumably the sites of nucleic acid in intact particles (Schramm *et al.*, 1955; Fraenkel-Conrat and Williams, 1955). Such preparations, and those dried while frozen (Stahmann and Kaesberg, 1955), also sometimes show particles that have broken transversely, with some fragments incompletely separated longitudinally and joined in their centers by threads, presumably nucleic acid. Positive evidence that such threads are nucleic acid was provided by Hart (1955), who found that treating virus preparations with sodium dodecyl sulfate in appropriate conditions produces rods with degraded ends, from which protrudes a core of material that is destroyed by pancreatic ribonuclease but not by trypsin or deoxyribonuclease.

C. *Fragmentation and Reassembly of Tobacco Mosaic Virus*

This work on the disruption of tobacco mosaic virus is relevant to the subject of virus multiplication for several reasons. First, it gives an indication of the relative roles of virus and nucleic acid, and secondly, it provides a picture of how the virus particles may be put together *in vivo*. All treatments that disrupt the virus particles lead to an immediate great loss of infectivity and most to the complete loss of this property. However, preparations disrupted by phenol (Gierer and Schramm, 1956) or by sodium dodecyl sulfate (Fraenkel-Conrat, 1956) can still be infective after all the characteristic rodlike particles have been destroyed; the infectivity is now clearly associated with much less stable material than intact virus particles, because it is lost in a day or so at 18° and very rapidly in the presence of ribonucleases. Such preparations consist largely and possibly exclusively of nucleic acid, and there is little reason to suspect that the nucelic acid alone is not infective. Per unit of phosphorus such preparations are only about one-hundredth as infective as intact virus particles, but the relative infectivity of the two types of preparation depends on the physiological state of the plants on which they are compared and on their relative concentrations (Bawden and Pirie, 1957b). Union with the protein clearly prevents the nucleic acid from being inactivated by many treatments *in vitro* and this fact makes it difficult to assess the true infectivity of the separated nucleic acid; it may be intrinsically as infective as intact virus but be unable to show this fact because so much becomes inactivated during the hazards encountered during and after inoculation to test leaves, hazards which the protein may well protect it against.

Treatment with phenol or sodium dodecyl sulfate, as with most other substances that separate the protein from the nucleic acid, denatures the protein, but alkali leaves the protein soluble and still able to react specifically with virus antiserum. We have already seen that protein fragments free from nucleic acid and with "molecular" weights of down to about 100,000 can be made by acid to reassemble into rods morphologically resembling virus particles. When these proteins are made to aggregate in solutions containing nucleic acid, whether from tobacco mosaic virus (Fraenkel-Conrat, 1956) or other sources (Hart and Smith, 1956), nucleic acid is incorporated into the rods, to the same extent as in normal tobacco mosaic virus and, apparently, in the form of a central core (Fraenkel-Conrat and Williams, 1955). The reconstitution of particles with the form and gross chemical constitution of the virus has been claimed to confer infectivity on mixtures of nucleic acid and protein, neither of which previously possessed it, but there is little reason to think that more has been achieved than either removing infective particles from the protein fragments, which act as inhibitors of infection, or than stabilizing infective nucleic acid, which otherwise would have become inactive before or during testing (Bawden and Pirie, 1957b).

Our picture of virus formation, however, does not necessitate the ability to "create" infectivity *in vitro*. It is enough that particles with the morphology and gross constitution of the virus can be put together, for *in vivo* the nucleic acid will presumably usually be active. The big blank space in our picture is in the early steps of particle formation, because particles broken down by alkali to what seem to be the basic protein subunits, with molecular weight around 18,000, are not reaggregated into rods *in vitro* by treatments that aggregate those of about 100,000. Also, of course, how the right amino acids get arranged in the right order to give the specific protein subunits, is something that can only be guessed at. It is a general problem of protein synthesis and one not specific to virus multiplication, and so, for a book on plant pathology, it will perhaps be enough to use some such cliché as "the necessary information is carried by the virus nucleic acid." We assume, then, that the intrusion of the virus nucleic acid into a host cell first changes the pattern of nucleic acid synthesis in the cell, so that more like itself is made, that this in turn changes the pattern of protein synthesis, so that the amino acids of the host cell are condensed into peptide chains of a new and specific form, and when these have built up to an adequate size they aggregate together in a spiral pattern with the nucleic acid, as *in vitro*, to give the characteristic stable rods.

D. *The Range of Anomalous Components in Extracts from Leaves Infected with Tobacco Mosaic Virus*

This picture of virus formation, largely derived from work on artificially fragmented virus, gains considerable support from the study and fractionation of extracts from infected plants. It has long been known that infective extracts do not contain a single type of anomalous particle, but a range of serologically related particles varying in size and infectivity, and that the small ones readily aggregate *in vitro* into long rods (Bawden and Pirie, 1945a). The smallest of these anomalous particles do not sediment when the sap is ultracentrifuged, they contain little or no nucleic acid, have a different electrophoretic mobility from the larger, nucleoprotein, particles, and in many ways resemble the fragments of about 100,000 molecular weight produced when tobacco mosaic virus is disrupted by alkali (Takahashi and Ishii, 1952, 1953). Are they stages in the synthesis of virus particles or degradation products? As these small particles do not occur in detectable amounts until the virus content of recently infected leaves is already considerable, they might seem to be breakdown products but this is far from conclusive (Commoner and Rodenberg, 1955; Bawden and Pirie, 1956). They never form a large part of the total anomalous particles, so if they are produced more or less simultaneously with large particles, in the same ratio that the two occur late in infection, then the small ones could not be detected until the virus content of leaves was considerable. Van Rysselberge and Jeener (1955) have provided evidence suggesting that the small particles are probably stages in synthesis rather than degradation products. They did this by experiments in which tobacco leaves infected for 3 days and in which the virus was increasing rapidly were exposed for short periods to $C^{14}O_2$, after which the leaves were macerated and the proteins in the sap separated into three fractions, normal leaf protein, the virus particles, and the small anomalous particles serologically related to the virus. The first and third fractions did not increase in amount during the course of the experiment, but the specific radioactivity of the third fraction increased by more than 60 times as much as did the normal leaf proteins. Hence, because the small anomalous particles were being synthesized rapidly, their total amount did not increase, but it can be assumed that they were being transformed equally rapidly into a different fraction that does accumulate, a role fulfilled by the rod-shaped virus particles.

It seems probable, then, that the small protein particles free from nucleic acid are intermediates in the synthesis of virus particles and that up to the stage of their formation, to molecular weights of 100,000 or so, they are produced separately from the nucleic acid. At this stage the two constituents come together and aggregate to give the large rod-shaped particles, by what mechanism is unknown, but nothing very subtle is required as the process can be simulated *in vitro* by acidification or exposure to ammonium sulfate solution. No ready-made protein rods are needed *in vitro* to act as "models" or starters for the process, and so there would be no need to postulate that *in vivo* the process depends on and demands the replication of an existing particle, even if there were no evidence that multiplication can be initiated by nucleic acid alone.

The protein intermediaries are readily detectable because of their serological relationship to the virus and because they are reasonably stable in expressed leaf sap. There is no such easy test for the nucleic acid, which does not react with virus antiserum, and which has only one specific identification tag, infectivity, to distinguish it from "normal" nucleic acid. Infectivity is lost almost immediately purified nucleic acid is added to leaf sap, and so the problems of identifying free virus nucleic acid in leaf extracts are considerable. Commoner (1953) reported that the insoluble nucleic acid increases in amount in infected tobacco leaves before tobacco mosaic virus occurs in them in large quantities and it then decreases as the amount of virus obtained in sap increases, suggesting that the increase might be in virus nucleic acid. By grinding leaves in liquid nitrogen at the time when the virus is increasing rapidly, Cochran and Chidester (1957) state that they obtained extracts from which infective nucleic acid preparations could be made. As there is no reason to assume this came from disrupted virus, it provides presumptive evidence of the virus nucleic acid occurring in the leaves free from its protein, and so studies with each component independently suggest that the two are synthesized separately. The most striking effect from combining the two that has yet been noted is to stabilize the fragile nucleic acid against many things that inactivate it when it is free. However, the fully assembled particles may well have many properties different from those of the separated components; indeed, the union may also stabilize the protein, for the rod-shaped particles assembled *in vitro* from protein alone disaggregate when the pH is raised more readily than do reassembled rods that also contain nucleic acid.

E. *Infections with Viruses Other than Tobacco Mosaic Virus*

No other plant virus has been studied to anything like the same extent as tobacco mosaic, so it is impossible to be sure that the picture already drawn applies at all generally. However, there are features of several that suggest a similar behavior. For example, X-ray analysis of other elongated and spherical viruses shows that these are composed of repeating units comparable to those in tobacco mosaic virus. Also, there is good reason to think that the nucleic acid is carried internally in some of them. Potato virus X is readily hydrolyzed by proteolytic enzymes, whereas the nucleic acid, which is readily hydrolyzed by pancreatic ribonuclease when it is freed from the protein, is unaffected by this enzyme when intact particles are incubated with it (Bawden and Kleczkowski, 1948).

The spherical virus, turnip yellow mosaic, also seems to carry its nucleic acid inside a protein shell. Extracts from infected plants contain two kinds of anomalous particles, of the same size but of different weights and so they can be separated by differential centrifugation (Markham and Smith, 1949). One kind of particle consists of protein only, the other of nucleoprotein, and only the second type is infective. Both kinds of particles have the same antigens and the same electrophoretic mobility, properties that would mainly reflect superficial characters, so that it seems that the nucleoprotein particles have only protein on their outsides. In plants kept in air containing $C^{14}O_2$ Jeener (1954) found that the particles composed of only protein acquired a specific radioactivity several times greater than the nucleoprotein particles, a result that seems to preclude the idea that the protein is a virus degradation product and suggesting that it is more probably an intermediary in the formation of virus particles. However, as a considerable proportion of the total anomalous protein in extracts from infected plants occurs as protein-only particles, even when the virus content of leaves is no longer increasing rapidly, it seems doubtful that all such protein does become incorporated into completed virus.

There is evidence with most virus diseases that have been studied in any detail that extracts of infected leaves contain more than one specific product of infection. In infections with tomato bushy stunt virus, the multiplicity of products has been shown by serological tests using the gel-diffusion technique (Kleczkowski, 1957); in infections with potato virus X, by the occurrence of more than one component detectable by electrophoresis (Bawden and Kleczkowski, 1957); and in infections with the Rothamsted tobacco necrosis virus by the differences in the ratio of infectivity to serological activity between different virus preparations (Bawden and Pirie, 1945b, 1957a). The nature of some of these products has still to be determined, but those that are serologically active can be assumed to be protein or nucleoprotein. Infectivity so far has always been associated with nucleoprotein or nucleic acid, but not all nucleoprotein particles with the gross morphology and chemical constitution of infective particles are infective (Bawden and Pirie, 1945a, b, 1956, 1957a).

The origin and biological significance of most of these noninfective particles is obscure. Some of them, as we have already seen, are possibly stages in the synthesis of virus particles, but others may equally represent breakdown products of virus or inactivated virus, and others may be concomitant products of virus synthesis that were never destined to become infective. There is no need to assume that the multiplication of viruses must inevitably run smoothly from the initiation of the infection process in a cell to a perfect end product of identical particles and with no waste. In the course of making such large particles there must be many opportunities for "errors" in assembly and for components to be missing at critical moments. The protein-only particles in plants infected with turnip yellow mosaic virus may simply mean a failure of nucleic acid synthesis to keep pace with protein synthesis, or that the protein subunits can fall into place and become assembled in ways that preclude the inclusion of nucleic acid. Nor should virus multiplication be regarded simply as a one-way process leading to complete particles that are then unchangeable entities. Nucleoprotein metabolism is a dynamic process in which the amount of any type at any time represents the balance between synthesis and degradation, and the occurrence and detection of particles, whether infective virus, synthetic intermediaries, or fragmented virus, will depend primarily on their stability in the cells and in the extracts from them. Leaf extracts contain nucleases that rapidly inactivate tobacco mosaic virus nucleic acid, but their proteases do not readily hydrolyze the protein fragments; it is, therefore, not surprising that free protein is easily detectable but the nucleic acid not. Tobacco leaf extracts also contain systems that can inactivate the Rothamsted tobacco necrosis virus without disrupting it and so the occurrence in them of noninfective nucleoprotein is also not unexpected (Bawden and Pirie, 1945a, 1957a).

III. Analogies with Bacteriophages

That the speculative picture of the *in vitro* formation of tobacco mosaic virus, drawn in Section II, may not be too far from reality is suggested by what is known of the behavior of T2 bacteriophage. Also, as these two such chemically and morphologically dissimilar viruses share so many common features, it is reasonable to think that the picture, at least in outline, will also apply to many others. So much has been found out about T2 that it is worth considering in some detail to seek suggestions about stages in virus multiplication that are still obscure with viruses of higher plants.

T2 is a tadpole or sperm-shaped particle consisting of almost equal amounts of protein and deoxyribonucleic acid. The protein is mainly or wholly confined to the tail and to a membrane around the head from which the nucleic acid can be removed by osmotic shock to produce a ghostlike particle. The particles attach themselves to host bacteria by their tails, at first loosely but then firmly. The power of specific attachment to the bacteria is possessed by the protein "ghosts," which also contain most or all of the specific antigens of the virus. Infection occurs by the tail dissolving a hole in the cell wall, through which the nucleic acid and little else passes, and the "ghosts" remain attached to the outside of the bacteria.

This ability to infect its host unaided is perhaps the most striking difference between the bacterial viruses and those that affect higher plants, all of which seem to need a vector to pierce their host's cuticle before they can infect. Howver, once inside a leaf, the plant viruses do move from cell to cell and their protein may play a part in this movement analogous to that of the bacteriophage tail. Also, there are many components in leaf cells, plastids, nucleus, microsomes, and the like, which are as large or larger than bacteria; it is not yet known where in cells plant viruses multiply, but if it is in one of these components, the protein of the virus may aid its entry or at least serve the function of getting the nucleic acid to the cell component unharmed by the cell nucleases. Some leaf cells are killed by infection, for example the mesophyll of *Nicotiana glutinosa* by tobacco mosaic virus, and by analogy with the ability of bacteriophage ghosts to dissolve bacterial cell walls, it is reasonable to suspect the virus protein to be responsible.

There is no direct evidence that the protein of tobacco mosaic virus serves any function other than safeguarding the nucleic acid, but that it may combine with something in plant cells as an essential step toward infection is suggested by the ability of protein fragments to inhibit infection by normal virus (Bawden and Pirie, 1957b). There is a similar specific inhibition by virus inactivated with ultraviolet radiation, a treatment that probably mainly harms the nucleic acid (Bawden and Kleczkowski, 1953). However, the inhibition by neither of these materials is as great as that by many other substances not chemically related to the virus protein, and knowledge of the mechanisms underlying the inhibition of infection is too slight for any specific interpretation.

That plant viruses do change their state drastically when causing infection is indicated by the results of experiments using ultraviolet radiation and pancreatic ribonuclease. First this is indicated by the behavior of irradiated virus preparations. Irradiation inactivates, but the residual infectivity of irradiated preparations of some viruses is greater when the inoculated plants are kept in the light than when they are kept in darkness. This phenomenon, called photoreactivation, is shown particularly strongly by potato virus X, which is, therefore, a suitable test object for studying it in detail (Bawden and Kleczkowski, 1955). Experiments with inoculated plants put in the light or dark at various times and for various periods after inoculation indicate three stages in the condition of the virus. The first is the stable state of the particles in the initial inoculum, in which they are not susceptible to photoreactivation; a few particles move from this to the second state within 15 minutes at 20° C., but most need about 30 minutes and a few as long as 2 hours. In the second state, the virus is photoreactivated by a few minutes' exposure to light, and then infection proceeds normally; the second state is unstable, however, and if photoreactivation does not occur within an hour or so, the virus is permanently inactivated.

That a change to a transient unstable state is not peculiar to irradiated virus particles is indicated by exposing leaves to ultraviolet radiation before they are inoculated with normal virus. Irradiation makes leaves insusceptible to viruses to which they would otherwise be susceptible, but the acquired resistance is only temporary provided the irradiated leaves are kept in the light. Even such stable viruses as tobacco mosaic, however, do not remain infective when introduced into leaves during their resistant period; their inability to persist for the few hours before the leaves again become susceptible strongly suggests that the particles present in the inoculum must have lost their normal resistance to inactivation by cell constituents (Bawden and Kleczkowski, 1952).

In speculating on the nature of the change that makes infecting virus particles less stable than they are *in vitro*, it may be significant that the time after inoculation of leaves—during which irradiated potato virus X remains susceptible to photoreactivation—is approximately the same as that during which ribonuclease can prevent infection from becoming established. This enzyme is a powerful inhibitor of infection when inoculated along with any virus with which it has yet been tested (Loring, 1942; Kleczkowski, 1946); it hydrolyzes virus nucleic acids when separated from their proteins, but does not destroy intact virus particles *in vitro*. When applied to leaves after they have been inoculated with virus, it inhibits infection less than when it is contained in inocula and its effect decreases with increasing delay between inoculation and its application; with a tobacco necrosis virus (Bawden and Harrison, 1955) and potato virus X (Bawden and Kleczkowski, 1955) it ceased to have any effect within about 2 hours of inoculation when applied to the leaf surface, and with tobacco mosaic virus within 6 hours when infiltrated into tobacco leaves (Casterman and Jeener, 1955). The mechanism responsible for inhibiting infection is uncertain, but clearly some early step in the cycle of events is prevented and there seems no more likely explanation than the one advanced by Casterman and Jeener (1955) that the enzyme hydrolyzes the nucleic acid. For this to happen, the virus nucleic acid must be freed from the protein, but it seems to be exposed for only a short time before it is again secure against the enzyme, presumably because it has found protection by combining with some cell component. This explanation is wholly convincing, but unfortunately is not established and others can be offered; for example, pancreatic ribonuclease also inhibits infection of *Rhizobium* sp. by bacteriophage, which there is no reason to think contains ribonucleic acid, and this it seems to do by preventing the phage particles from attaching themselves to the host cells (Kleczkowski and Kleczkowski, 1954).

However, further circumstantial evidence for the idea that virus nucleic acid and protein become separated as an initial step in infection comes from experiments in which leaves were exposed to ultraviolet radiation at intervals after inoculation. For some time after inoculation, the sensitivity of tobacco mosaic virus to inactivation remains constant, but after a lag period that varies with different strains, from about 2.5

hours with strain U2 to 5 hours with the type strain, the sensitivity decreases (Siegel and Wildman, 1956). There is no such lag period when inoculations are made with infective preparations of nucleic acid instead of intact virus particles; as Fig. 2 shows, both strains then behave alike and their resistance to inactivation begins to increase almost immediately after inoculation (Siegel *et al.*, 1957). The reason for the increased resistance with time can only be guessed at, but it probably means that the infecting virus has now become associated with other materials that absorb radiation of wavelength 2,537A. or that the amount of such materials (purines and pyrimidines ?) has been increased as a result of infection. Whatever the reason, the fact that inocula of nucleic acid become resistant sooner than those of intact virus is, as suggested by Siegel *et al.* (1957), *prima facie* evidence that the difference is the time taken *in vivo* for the protein of the intact virus to be removed from the nucleic acid. These authors also point out the possible significance in this context of the fact that the nucleic acid of strain U2, which has the shorter lag period *in vivo*, separates from its protein more rapidly than that of the type strain when treated with detergent *in vitro*. There is one difficulty in accepting this explanation for the lag period; if nucleic acid from the type strain has a start of several hours over intact virus in initiating infection, lesions might be expected to appear earlier in *N. glutinosa* with inocula of nucleic acid than with intact virus, whereas

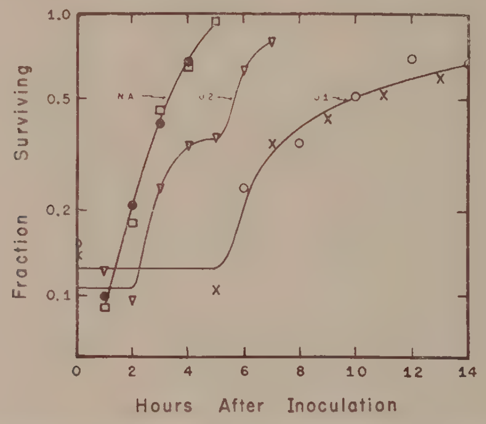

FIG. 2. Ultraviolet light survival of infective centers as a function of time after inoculation. Dose of UV: intact U2 and nucleic acid, 90 seconds; U1, 5 minutes.

KEY: (solid circle) U1 nucleic acid; (square) U2 nucleic acid; (triangle) intact U2; (open circle) and ×, intact U1.

on the many occasions on which I have inoculated the two to opposite half leaves, I have never detected any consistent differences in the length of time taken for lesions to become visible. However, Schramm and Engler (1958) state that virus multiplication becomes detectable in inoculated tobacco leaves 10–12 hours sooner when the inoculum is nucleic acid than when it is intact virus.

The only other virus with which similar experiments have been done is one of those causing tobacco necrosis; like tobacco mosaic virus it retains its initial susceptibility to inactivation by ultraviolet light for an hour or more after inoculation to leaves, but then its susceptibility decreases (Bawden and Harrison, 1955). Infective preparations of nucleic acid from this virus have not yet been made so it is unknown whether using nucleic acid alone would abolish the lag period and allow the virus immediately to reach some haven or initiate the synthesis of ultraviolet-absorbing substances.

Although there is good reason for concluding that plant viruses undergo some change analogous to the separation of the protein and nucleic acid of T2 bacteriophage as its first step in infecting, there is no evidence to suggest whether this event happens as a preliminary to plant viruses entering their host cells or when they have entered and met some specific cell constituent. The next steps with plant viruses are wholly wrapped in obscurity and for this part of our picture we must rely almost totally on knowledge derived from work with bacteriophages. For the first half of the period between infection and lysis of bacteria, no bacteriophage particles are detectable in artificially disrupted cells and neither serology nor electron microscopy detects any material that can be related to the bacteriophage. During this "eclipse" phase, however, much is going on. The nucleic acid metabolism of the bacterium alters and the genetic determinants of the bacteriophage start to multiply almost immediately after infection occurs. The protein metabolism also changes, but whether coincident with or after the synthesis of bacteriophage nucleic acid is uncertain. The reappearance of infectivity coincides with the first occurrence of tadpole shaped particles early in the second half of the period between infection and lysis, and from then on infectivity and the number of such particles increase linearly until the bacterium is lysed. Shortly before these mature particles occur, material with the serological specificity of the bacteriophage becomes detectable and electron microscopy reveals, first, round particles of a size similar to that of the bacteriophage heads, and, later, ones with tails but still lacking nucleic acid and infectivity. These are almost certainly precursors of mature bacteriophage, and analogies between them and the protein-only particles that occur in extracts from virus-infected leaves are too obvious to need stressing. When bacteria are simultaneously infected by two strains of T2, the first-formed infective progeny combine characters from the two, indicating that genetic recombinations occur, not by exchange of material between mature particles, but when the nucleic acid is multiplying before it becomes incorporated in its protein. Rather less than half of the phosphorus contained in infecting bacteriophage is transmitted to the offspring, but in what chemical state it is handed on, and what proportion of the progeny contains parental material, is unknown. The protein is derived mostly from materials in the medium at the time the bacterium is infected, but some comes from low molecular weight material already in the cell. The nucleic acid derives from a pool of compounds, either bases, nucleotides, or polynucleotides, which less rapidly equilibrate with the medium than the materials making the protein, a pool that is maintained partly by the breakdown of pre-existing bacterial components and partly by new synthesis from the medium (Hershey, 1953; Luria, 1953).

As the nucleic acid of tobacco mosaic virus can alone initiate infection, and protein-only particles that combine with nucleic acid occur in extracts from infected leaves (Commoner, 1957), there are enough similarities between its behavior and that of T2 early and late in the infection cycle to assume that intermediate events will not be entirely dissimilar. In other words, it seems reasonable to assume that the intrusion and replication of the virus nucleic acid establish new patterns of protein synthesis in the plant cells, which for a time will contain no mature particles; these will form not by the replication of existing particles, but by a distinct process of assembling component parts that have either multiplied themselves or been synthesized *de novo*. The mature particles are probably largely static and divorced from further activity in the cells; variants, distinguishable from the initially infecting strain by changes in pathogenicity or some other property, probably arise when the nucleic acid is multiplying, presumably because of failures to copy the precise pattern of the infecting type. Some of the changes now thought to be mutations may well result, not from genetic changes, but from already existing determinants separating and recombining in different combinations. There has been little work yet on genetic recombination in plant viruses, but Best and Gallus (1954) have produced substantial evidence that, when different strains of tomato spotted wilt virus are multiplying together in the same plants, progeny are produced that combine characters from the different strains.

The idea has often been advanced that virus multiplication entails the change of a normal host component into virus, in a manner analogous to that in which trypsin converts its inactive precursor, trypsinogen, into active enzyme. We have seen that the components of bacteriophage are built up from small units and that no major block of normal host protein or nucleic acid is incorporated in them. Similarly, although Wildman *et al.* (1949) claim that tobacco mosaic virus is produced at the expense of a major protein component in tobacco leaf cytoplasm, other workers (Commoner *et al.*, 1952; Bawden and Kleczkowski, 1957) have failed to confirm this. Indeed, Commoner (1953) has claimed that the protein is not even made from the common pool of cell amino acids, but that its amino acids are synthesized *de novo* from the free ammonia in the host.

Indirect evidence for the synthesis of nucleic acid, or its intermediates, before any new virus particles become detectable, is provided by the continuing increase in resistance of multiplying virus to inactivation by ultraviolet radiation with increased time after infection. This increase occurs for a few hours without the inactivation curve changing from the exponential type to be expected if only one target in infected cells needed to be hit to prevent multiplication, but it then becomes the multitarget type, suggesting that the preliminaries to replication of the nucleic acid have been completed and that there are now additional infective centers (Siegel and Wildman, 1956). This again happens long before any virus multiplication is detectable by infectivity assays, serological tests, or electron microscopy; indeed, it happens while the amount of virus recoverable in extracts from inoculated leaves is apparently falling, but the difficulties in interpreting such falls and other phenomena that superficially suggest something like the "eclipse" phase in bacteriophage infection are considerable. These difficulties are considered in the next section.

IV. FACTORS AFFECTING VIRUS MULTIPLICATION

A. *The Normal Multiplication Curve*

When leaves are inoculated, washed to remove unattached virus, macerated at various intervals, and the extracts assayed for their virus content, it is a simple matter to determine the rate at which virus increases in the leaves. Provided conditions are kept constant, reasonably reproducible results are obtained with any given inoculum and host. After an interval ("latent period"), which depends on the temperature, the virus content of the inoculum and on the sensitivity of the method of testing, new virus becomes detectable, and the infectivity of successive extracts then usually increases rapidly. The infectivity of successive extracts made during the latent period, however, decreases (Harrison, 1956a), and at Rothamsted we also find fewer virus particles detectable by electron microscopy at the end than at the beginning of the latent period, but the results published by Steere (1952) show no such fall. Increasing temperature up to an optimum, which differs for different viruses, shortens the latent period and initially increases the rate at which new virus accumulates, but this optimum is often above the temperature at which the maximum virus content of leaves can be obtained. When the host reacts with a sharply localized necrotic reaction, virus usually increases rapidly only while the lesions are developing, but otherwise the virus content continues to increase for several days, first exponentially and then proportionally less rapidly as it reaches a maximum that differs greatly with different viruses and hosts. Tobacco mosaic virus in tobacco at about 20° reaches a high maximum, which then stays reasonably constant for long periods; others such as potato virus Y reach a lower maximum, which is also maintained, but others, of which alfalfa (lucerne) mosaic virus is a striking example, having reached their maximum then rapidly decline in amount (Ross, 1941).

It is easier to get such results than to interpret them. The leaf is a multicellular system of which only some of the epidermal cells become infected at the time of inoculation. Also, the fate of the inoculum is uncertain; washing removes about 99.9% of tobacco mosaic virus sprayed on to leaves, but only 94% of that inoculated by rubbing (Yarwood, 1952b; Harrison, 1956a). Most of the particles remaining on rubbed leaves, however, seem not to infect, for about a million are retained for each local lesion that develops. Measurements on the infectivity of leaf extracts made at different times after inoculation cannot alone tell whether the fall during the latent period has any significance in the course of events that lead to virus multiplication. Nor can they tell whether the first rise in the infectivity of extracts identifies the first crop, or generation, of new virus; or for what period the virus content of single cells continues to increase, and whether the period of exponential increase of virus mainly indicates increase of virus per cell or increasing numbers of cells becoming infected.

Experiments with tobacco mosaic virus in *Nicotiana glutinosa* suggest that the decrease in infectivity of extracts during the latent period is irrelevant to the process of infection and that it probably occurs because surplus inoculum is being inactivated. The infectivity of extracts begins to increase only shortly before lesions become visible, when many mesophyll cells are already infected and dying. Only epidermal cells are likely to become infected directly from the inoculum, so the first generation of infective material seems to pass undetected, for virus from the inoculum must presumably have multiplied in the epidermis and its progeny spread from there and increased in the mesophyll before the infectivity of the extracts begins to increase.

Better evidence that the first cycle of multiplication passes undetected comes from exposing inoculated leaves to ultraviolet radiation at different times after inoculation. Moderate exposures can be expected to inactivate virus in the epidermis but not in deeper tissues, and by finding at what interval after inoculation irradiation halves the number of infections, the mean time for infection to spread from epidermis to mesophyll can be found. It is approximately half the latent period, or half the time taken for necrotic local lesions to become visible in *N. glutinosa* leaves inoculated with tobacco mosaic virus, so that it seems reasonably certain that only the second cycle of multiplication is detected (Harrison, 1956a).

Various explanations can be advanced for the failure to detect the first crop of virus formed in cells infected by the inoculation. The particles may be in some way immature, able to move to and infect neighboring cells, but not yet stable enough *in vitro* to provide an effective inoculum. Or they may all become firmly and specifically attached to "infection sites" in nearby cells and never become free in extracts. The simplest explanation, however, is that the first crop produced in the epidermal cells is too small to be detected by current inoculation methods; indeed, too small to balance the inactivation of the excess inoculum. As already said, about a million virus particles must be rubbed on leaves to produce one local lesion, and this is the order of size of the number of particles estimated to be present in mesophyll cells fully infected with either a tobacco necrosis virus (Harrison, 1956a) or tobacco mosaic virus (Nixon, 1956). It is not surprising, then, that the virus first formed in inoculated epidermal cells should pass undetected in leaf extracts, although there is enough to infect more than 100 nearby cells. It is the crop from these secondarily infected cells that makes its presence felt in the extracts, and from then on the increases in infectivity will reflect both increase in the virus content of already infected cells and increasing numbers of cells becoming infected. In each cell, it is reasonable to assume that the sequence of events postulated for the initially infected cells will be re-enacted, but the establishment of infection will probably be better assured; if virus introduced at inoculation becomes inactivated in the epidermal cells it is not replaced, whereas the supply for secondarily infected cells will be almost unlimited.

There has been little work on the virus content per cell compared with that on the virus content of a volume of leaf, but Harrison (1956a) concluded that the amount of tobacco necrosis virus in individual leaf cells probably continues to increase at 22° until about 36 hours after they become infected. As the virus content of leaf extracts continues to increase for a week or more, the continuing increase comes from the virus invading and multiplying in new cells. With viruses that kill their host cells, the reason for reaching a maximum virus content is obvious, but with those that do not, this may happen for different reasons; multiplication may stop because the supply of metabolites is exhausted, or multiplication may continue but be balanced by degradation of already produced virus. It seems that viruses like alfalfa mosaic, whose amount decreases rapidly after reaching a maximum, must be degraded, and some inactivating system may develop in cells as a consequence of infection. Also, at 30°, there seems little doubt that the Rothamsted tobacco necrosis can both multiply and be inactivated, and although unequivocal evidence of inactivation is difficult to get at lower temperatures, the results are compatible with the idea that both processes proceed simultaneously (Harrison, 1956b). Even tobacco mosaic virus, which in short-term tests with radioactive nitrogen seemed to be wholly stable (Meneghini and Delwiche, 1951), is inactivated at higher temperatures, and the virus content of leaves slowly decreases when plants are kept at 36° (Kassanis, 1957c). In this context it is perhaps significant that only viruses that are unusually stable *in vitro* reach and maintain high concentrations *in vivo*, and the low maxima achieved by unstable viruses may not reflect any less ability to multiply but the fact that they are more readily broken down *in vivo*.

So far we have considered virus multiplication only in inoculated leaves, but this is only one feature of the story, for in many plants viruses become systemic and spread from inoculated leaves to most of the vegetative tissues that were still growing when the plant became infected. This is no place to review the large literature on virus movement through plants, and it will suffice to say that, although the direction of movement through the vascular tissue is correlated with the movement of elaborated foods, there is no reason to think that there is a mass flow of virus from inoculated leaves to other parts of plants. Nor, because of its speed, is there any reason to think that it occurs along a concentration gradient built up by continuing virus multiplication, such as happens in cell-to-cell spread through a leaf. Rather it seems that a few particles, whether stable and resembling those that are infective in extracts or unstable (nucleic acid?) is unknown, move over large distances in the food stream and then set up new infections, where, presumably, the events already described for inoculated leaves are repeated. In leaves that are already developed when they become infected, virus increases to a maximum content comparable to that in leaves rubbed with concentrated inocula. Some viruses, such as tobacco mosaic and potato Y, also attain similar concentrations in leaves that develop after plants become systemically infected, but others, and particularly those that initially cause severe ring spot diseases from which the plants later recover, occur in the symptomless leaves at only a fraction of the concentration they attain in inoculated leaves or in systemically infected ones that develop severe lesions (Wingard, 1928; Price, 1936; Bennett, 1949). Cells that become infected soon after they are differentiated seem either to fail to produce some materials essential for the synthesis of these viruses in the same amounts as they occur in cells that differentiate uninfected, or to produce some mechanism that inactivates the virus. Whatever is the restricting factor, it is overcome in plants that have suffered and recovered from diseases caused by dodder latent mosaic virus when these plants are infected with tobacco mosaic or tobacco etch virus. The doubly infected plants then again develop symptoms similar to those of their initial reaction, and the amount of dodder latent mosaic virus in the leaves immediately increases and remains indefinitely at a high level (Bennett, 1949).

It is rare for viruses to occur in pollen mother cells or egg cells and many do not occur in stem apical meristems (Morel and Martin, 1952, 1955; Kassanis, 1957b), but whether this is because such cells do not support virus multiplication, or because the viruses are prevented from reaching them, is unknown.

B. *Effects of Changing the Environment*

The prime factor that determines whether a given plant acts as a host for a given virus is, of course, its inherent ability to sustain the virus, but nurture can be almost as important as nature, and a plant may be a favorable host in one physiological state but unfavorable in others. With the seasonal variations in growth of plants experienced in northern latitudes, it is necessary only to inoculate plants throughout the year to see how profoundly changes in the environment affect the ease with which plants contract infection, especially with mechanically transmitted viruses. Some of these, for instance those causing tobacco necrosis, are almost difficult to maintain in tobacco during the summer but will rapidly kill inoculated leaves in winter. Perhaps the extreme example of seasonal variation is provided by cucumber mosaic virus in some varieties of French bean, which between October and March in England are suitable hosts for local lesion assays, but between April and September produce no lesions and no virus is recoverable from inoculated leaves (Bhargava, 1951). We are still far from understanding what features of plants affect their susceptibility to infection and the extent to which viruses multiply when infection has occurred, and different viruses and hosts will no doubt behave differently. However, enough has been done on altering the growing conditions of plants to show that almost any change that affects their physiology affects their behavior toward virus infections, and that not all conditions that predispose plants to infection are also those that most favor the accumulation of viruses in large quantities. Here it will be simplest to consider changes in nutrition, light intensity, and temperature separately, but normally, of course, these will not vary independently.

1. *Changes in Nutrition*

The appearance and growth of plants respond rapidly and strikingly to changes in the supply of water and nutrient and these changes are reflected in varying susceptibility to viruses. Little has been done on variations in water supply, but Tinsley (1953) found that *Nicotiana glutinosa* plants that received unlimited water produced 10 or more times as many local lesions when inoculated with various viruses as did plants watered enough only to prevent wilting. The differences in susceptibility to infection produced by differential watering were decreased by incorporating an abrasive in the inoculum or by growing plants under shade, and as increased watering produces plants with more succulent leaves and thinner cuticles, much of its effect may be attributable simply to the fact that the leaves are more easily damaged so that rubbing produces more entry points for virus particles.

The effects of differential feeding with nitrogen, phosphorus, and potassium, on susceptibility to infection as measured by the number of infections produced per unit area of leaf, are smaller than those of differential watering. However, their effects on the extent to which viruses increase in systemically infected leaves is considerable. In general, applications of nutrients that increase plant growth also increase susceptibility to infection by mechanical inoculation and they affect virus production in two ways, by increasing the amount of virus per unit of leaf and by increasing the total amount of tissue that becomes infected; the total amount of tobacco mosaic virus in tobacco plants receiving supplements of nitrogen and phosphorus that increased growth greatly was 40 times that in plants deficient in these elements and the virus content of sap was doubled. The content of potato virus X in sap from potato leaves, however, was not consistently increased by fertilizers (Bawden and Kassanis, 1949a,b). The concentration of cucumber mosaic virus in spinach was increased by additions of major nutrients as long as these also increased plant growth, but not beyond (Cheo *et al.*, 1952). Little work has been done with trace elements, but the content of tobacco mosaic virus in tobacco leaves was increased by additions of zinc that also increased plant growth (Helms and Pound, 1955), whereas it was greater in leaves deficient in manganese than in leaves adequately supplied with this element (Welkie and Pound, 1958).

In cultures of tobacco tumorous tissues, in which the concentration of tobacco mosaic virus is only about $\frac{1}{30}$ of that usual in leaves, the virus concentration was not correlated with the rate at which the tissues grew; it was not increased by increasing the amount of nitrogen in the medium, and was decreased by additions of phosphorus that increased the growth of the tissues (Kassanis, 1957a).

2. *Changes in Illumination*

Plants grown in high light intensity have tougher leaves with thicker cuticles than those grown in shade, and this may explain, at least in part, the greater susceptibility to infection of plants shaded before they are inoculated (Samuel *et al.*, 1935; Bawden and Roberts, 1947). However, there are almost certainly other factors involved, because some viruses, tomato bushy stunt for example, not only cause more local infections but also spread more readily through inoculated leaves and become more fully systemic in tomato plants kept shaded both before and after inoculation, than in plants grown in a high light intensity. Also, increases in susceptibility to infection equal to those caused by prolonged growing in shade are produced when plants are kept in darkness for a day or so before they are inoculated, and this treatment produces no obvious effect on the fragility of the leaves (Bawden and Roberts, 1948). Hence, differences in the composition of cells that have been illuminated differently are probably concerned in determining the ease with which viruses become established in cells, but which of the many changes produced by decreasing the light intensity or putting in darkness increases the likelihood of establishment is unknown.

With most viruses the effect is solely in predisposing plants to infection and shows by a given inoculum producing more local lesions per unit area of leaf treated before inoculation. Shading or putting in darkness after inoculation does not increase the number of lesions. Indeed, with some viruses, putting plants in the dark after inoculation decreases the number, usually only slightly, although different viruses behave differently and Best (1935) found that tobacco leaves inoculated with tomato spotted wilt virus produced more lesions when kept in dim light for 2 days after inoculation than when kept in the glasshouse. Although of little importance with normal inocula, the illumination after inoculation is critical when inocula consist of viruses irradiated with ultraviolet light, for then, and particularly with potato virus X, inocula that produce few or no lesions in plants kept in the dark after inoculation may produce many in plants kept in the light. This phenomenon of photoreactivation has already been mentioned in Section III and here no more need be said except to comment that it illustrates particularly vividly the extent to which the physiological condition of the host can determine whether introduced particles become established and multiply. Presumably some light-sensitive system in the host cells acts to repair damage done to the nucleic acid by irradiation, but whatever the mechanism, there is the striking fact that particles which will infect and multiply in illuminated cells fail to do so in unilluminated ones.

The effects of light intensity on the extent to which viruses multiply in infected leaves have been less studied than have effects on predisposition to infection. It may well be that they are considerable, but differ widely with different viruses. Many viruses produce more severe lesions, both local and systemic, in plants grown under low than under high light intensities; these are mainly viruses that produce symptoms of the mosaic and ring spot type, and potato viruses X and Y also seem to occur in greater concentrations in the more severely affected plants. At least in detached leaves, however, tobacco mosaic virus reaches higher concentrations in leaves kept in the light and supplied with nutrients than in leaves kept dark. Viruses that cause "yellows" type symptoms, however, usually produce more intense symptoms in plants exposed to high than to low light intensities; there is no conclusive evidence about the concentration of such viruses, which are mostly not transmissible mechanically, but as some are more readily transmissible by insect vectors from plants kept in high light intensities they may also occur in these in greater amounts.

3. *Changes in Temperature*

Considerable as are the effects of changing light intensity on susceptibility to infection and virus multiplication, they are small compared to those of changing temperature. With temperature, too, it is even more obvious that conditions that increase the likelihood of healthy plants contracting infection are far from those that favor the most extensive accumulation of viruses in infected tissues. Here the effects will be briefly indicated, as a full account with references to the original papers has recently been published by Kassanis (1957d).

Keeping healthy plants at a high temperature much increases their susceptibility to infection by mechanically transmitted viruses, so that a given inoculum may give 10 or more times as many lesions in leaves of plants kept for 2 days at 36° before inoculation as in those kept at 20°. This predisposition to infection by high temperatures occurs with all viruses yet tested and the size of the response depends more on the age and physiological state of the plants tested than on the identity of the virus. By contrast, keeping plants at 36° after they are inoculated usually decreases the number of lesions obtained, but the size of the decrease differs greatly with different viruses. Some produce almost as many at 36° as at 20°, whereas others produce few or none. The ability of a virus to multiply and produce lesions in leaves at 36° is not correlated with its resistance to heat as usually measured by determining the temperature at which the virus is inactivated after 10 minutes' exposure. Two of the viruses listed in Table II, for example, have unusually high thermal inactivation points, but these fail to become established in plants kept at 36°, whereas tomato spotted wilt virus, which has the lowest thermal inactivation point determined, does.

TABLE II

EFFECT ON MEAN NUMBER OF LOCAL LESIONS OF KEEPING PLANTS AT 36° C. BEFORE OR AFTER INOCULATION WITH VARIOUS VIRUSES[a]

	Plants at 36° C. before inoculation for			Plants at 36° C. after inoculation for		
Virus and host	0	1 day	2 days	0	1 day	2 days
Rothamsted tobacco necrosis (90)[b] in bean	2	29	46	69	0	0
Tomato bushy stunt (80) in *N. glutinosa*	18	83	97	65	9	2
Cucumber mosaic (60–70) in tobacco	15	45	78	144	0	0
Tobacco mosaic (93) in *N. glutinosa*	32	98	111	25	18	19
Tomato spotted wilt (45) in tobacco	86	197	165	173	121	96

[a] Data provided by Dr. B. Kassanis.

[b] Figures in brackets are the temperatures at which the viruses become inactivated in 10 minutes *in vitro*.

This failure to become established in plants at high temperatures cannot be correlated with any specific property of viruses, because some strains of one virus, for instance of cucumber mosaic (Hitchborn, 1956), become established in plants at 36° and others do not. It seems, though, that it is more common with spherical viruses, which have a higher nucleic acid content and a smaller Q_{10° of thermal inactivation than viruses with elongated particles, than with elongated viruses. Many spherical viruses cannot maintain themselves, even in tissues in which they are already established, when infected plants are kept continuously at temperatures around 36°, and exposure to high temperatures is the one therapeutic treatment that has yet been discovered and applied to produce virus-free lines of clonal varieties the stocks of which had become wholly infected. This inactivation is easily followed by assaying extracts from leaves at intervals after infected plants are placed at high temperatures. For example, when tomato plants systemically infected with tomato bushy stunt virus are placed at 36°, the infectivity of leaf extracts falls to about one-tenth of that at the start within 4 days, and to one-hundredth within a week. This rapid fall of infectivity is not accompanied by any corresponding fall in concentration of specific antigen in the sap, suggesting that changes in the nucleic acid are probably responsible for the inactivation and that the protein moiety is initially not greatly altered. With prolonged exposure to 36°, however, the virus antigens also disappear.

That virus multiplication is a dynamic process, with the concentration of virus at any one time representing the balance between synthesis and degradation, has already been suggested. The rates of all biological processes depend on temperature and are increased by increasing temperature up to an optimum. Different processes have different temperature optima; and it seems that altering the temperature at which plants are kept alters the relative activities of systems that are responsible for synthesis and of those that inactivate viruses. Table III shows that increasing the temperature at which French bean plants are kept up to 22° increases the rate at which a tobacco necrosis virus increases in newly inoculated leaves, whereas increases above 22° decrease it. Clearly up to 22° the rate of multiplication is increased by increasing temperature, and there is other evidence indicating that it is probably increased by increasing the temperature still further. Effects on rate of multiplication, however, are obscured by effects on rate of inactivation, which become increasingly important as the temperature and the virus content increase. This is readily shown by taking bean plants that have spent 2 days after inoculation with the tobacco necrosis virus at 22°, and so already have a considerable virus content, and then

TABLE III
THE EFFECT OF TEMPERATURE ON THE RELATIVE CONTENT OF ROTHAMSTED TOBACCO NECROSIS VIRUS IN INOCULATED FRENCH BEAN LEAVES[a]

	Relative virus content at time after inoculation		
Temperature (° C.)	23 hours	47 hours	71 hours
10	1	3	37
14	2	422	3,935
18	31	3,875	33,750
22	208	19,100	158,000
26	79	3,015	7,550
30	6	97	163

[a] Data provided by Dr. B. D. Harrison.

placing them for a day at 30°; although, as Table III shows, this virus can multiply at this temperature, such plants will then contain less than before they were put at 30°. Although this virus may be more susceptible to *in vitro* inactivation than most others, there is no need to think that it behaves in a qualitatively different manner. Even such a stable virus as tobacco mosaic virus can be inactivated at 36° C. (Kassanis 1957c) and most of the viruses that have been studied, although they multiply more slowly below than above 20°, ultimately reach higher concentrations at the lower temperatures.

Knowledge of the changes produced in leaves by changing temperature, and of the cellular activities that affect virus establishment and multiplication, is too slight for any conclusive explanation of the apparent paradox that exposure to 36° should be both a therapeutic treatment for virus-infected plants and the most effective treatment known for increasing the susceptibility of healthy plants to infection. However, if, as it has already been stated seems likely, infection of cells entails the initial step of virus nucleic acid separating from its protein, a possible explanation is suggested by the results of experiments comparing the relative infectivities of tobacco mosaic virus and preparations of its nucleic acid toward *Nicotiana glutinosa* in different physiological states.

From what has already been said in this section, it is clear the ability of normal virus preparations to infect varies greatly with the age and physiological activity of the inoculated leaves. Infection with separated nucleic acid of tobacco mosaic virus, however, depends even more on the condition of the host plants; the dependence is so great that the relative activities of the two types of inocula may be entirely different when compared on different batches of plants. Infections by the nucleic acid preparations occur much more readily in young, succulent leaves than in old, tougher ones, and an inoculum of nucleic acid that produces as many lesions as does a solution of normal virus in young leaves will produce very many fewer in old leaves. Similarly, when plants are placed at 36° before they are inoculated, the number of lesions produced by both types of inocula will increase, but proportionally the number produced by nucleic acid will increase much more. Further, when plants are placed at 36° after they are inoculated, the number of lesions formed by nucleic acid is decreased proportionally more than with normal virus inocula. Another point that may be relevant is that exudates from *N. glutinosa* leaves inactivate nucleic acid preparations much more rapidly *in vitro* at 36° than at 20°.

These observations all combine to suggest that success or failure in establishing infection depends on whether or not the nucleic acid survives through a seemingly hazardous period between inoculation and reaching some safe haven; the hazards are greater in leaves in some physiological states than in others and with inocula of normal virus the nucleic acid is partly but not wholly protected from inactivation by its protein. Maintaining leaves at a high temperature encourages protein degradation and produces other changes that may favor the survival of nucleic acid by decreasing the amount of substances that inactivate nucleic acid. If the inactivation of nucleic acid has a large $Q_{10°}$, however, inactivation will be much more rapid at 36° than at 20°, and so with a given amount of inactivators the chances of infection occurring will be less when inoculated plants are kept at the higher temperature. The hypothesis that high temperature decreases the amounts of inactivators in leaves, but increases the rate at which they act, adequately accounts for the different effects produced by pre- and post-inoculation exposures to 36°, but an explanation is not necessarily true because it is adequate and much more experimentation will be needed before it can be accepted.

Similarly, it is impossible to explain with certainty the fact that some viruses become established and can maintain themselves in plants kept at 36° whereas others cannot. If viruses fail to increase because their nucleic acid is inactivated at 36°, one obvious possibility is that the nucleic acids of different viruses differ in stability; others are that, in the course of infection, the nucleic acid from some viruses is exposed free for longer than that from others, or that the protein of some is less effective than that of others in protecting the nucleic acid from inactivators. In assessing the role of the protein it may be relevant to note two points. First, tobacco leaves contain a system that *in vitro* inactivates tobacco ringspot and the Rothamsted tobacco necrosis virus, neither of which becomes established in plants at 36° (Bawden and Pirie, 1957a); it does not affect intact tobacco mosaic virus but it readily inactivates the separated nucleic acid. Second, different strains of tobacco mosaic virus differ in their ability to infect *Nicotiana glutinosa;* weight for weight the type strain produces many more lesions than the rib grass strain, and Fraenkel-Conrat and Singer (1957) state that preparations made by combining nucleic acid from the rib grass strain with protein from the type strain were more infective than the original preparation of the rib grass strain, suggesting that its nucleic acid was now better protected against inactivation.

Temperatures of 36–40° are about as high as growing plants can tolerate for long periods, but leaves will stand brief exposures to higher temperatures. Yarwood (1956) found that immersing French bean leaves for some seconds in water at temperatures up to 50° greatly increased the number of lesions they produced when inoculated with any of several viruses. With some viruses, the hot water dip also increased the number of lesions when it was given some hours after the leaves were inoculated. Whether this increased susceptibility happens for reasons similar to the phenomena already discussed is not known, but the hot water dip seems less generally applicable than growing plants at temperatures around 36°; it did not increase susceptibility of plants other than Pinto beans that were tried, and it increased the susceptibility of beans to infection by fungi in addition to viruses.

In this review we are not primarily concerned with effects on symptoms, but these are sometimes related to virus multiplication and accumulation and so are relevant to our subject. Exposure to high temperatures decreases the severity of symptoms shown by many virus-infected plants, and this is usually correlated with a fall in virus content. Sometimes increasing temperature also changes the type of symptom, as with tobacco mosaic virus in *Nicotiana glutinosa,* in which the characteristic necrotic local lesions become larger as the temperature increases and, when plants are held at above 30°, change from necrotic to chlorotic (Samuel, 1931). When multiplication is studied in *N. glutinosa* leaves, the virus continues to increase for much longer in plants kept at over 30° than at lower temperatures and the virus content of leaves becomes much greater. At first sight, this seems to contradict the general thesis that virus accumulates more at low than at high temperatures, but the contradiction is apparent rather than real. There are two measures of virus content, amount per volume of leaf and amount per cell, and the two are not always correlated. The amount per leaf is greater at high temperatures because more cells become infected; infected cells do not die and the virus from each site of infection invades many more cells than at 20°. The amount of virus per cell, however, is much less at around 36° (perhaps this is the reason the cells do not die), and when plants that have been at 36° for some days after inoculation are then placed at 20° the virus content increases enormously within a day and the cells in the areas that were previously chlorotic die. Here again, some mechanism that at 36° determines the virus content of individual cells, either by breaking down virus or by inhibiting its synthesis, seems to become relatively inactive and unimportant as soon as the temperature is lowered to 20°.

For the sake of simplicity, tobacco mosaic virus has so far been written about in this section as though it were a single entity and changes in the temperature at which infected plants are grown as though they have only one type of effect. This is a gross oversimplification, for different strains of the virus differ considerably in their ability to become established and multiply in plants held at high temperatures. Most of what has already been stated applies to the type strain, which gives a severe mosaic with some leaf distortion in tobacco plants growing at about 20°, conditions in which it reaches very high concentrations. It causes less severe symptoms in plants held at 36°, not simply because it no longer reaches the same concentration, but also because it becomes partly superseded by less virulent strains that are able to reach higher concentrations at 36° than the type strain. It was by keeping infected tissues at high temperatures that Holmes (1934) isolated his "masked" strain, which produces slight or no symptoms in tobacco plants at 22°. Comparable effects have since been described by several people (Johnson, 1947; Sukhov, 1956; Mundry, 1957; Kassanis, 1957c), and there is little doubt that the strains with changed virulence are produced in the inoculated plants while they are at the high temperatures and are not selected from a mixture in the initial inoculum. Some workers have suggested that the high temperatures actually cause the new types by increasing the "mutation" rate of the virus, but for this there is no conclusive evidence. In plants kept at 20° such variants may also occur but then they would not be specially favored and so would be obscured by the infecting type; variants better able than the infecting type to survive or invade new cells at 36°, however, would be greatly favored and would soon become dominant in plants at the high temperature. Mundry (1957) states that a day at 35° is all that is needed to obtain new variants and that most are produced when the day of treatment is immediately after inoculation.

Kassanis (1957c) noticed two kinds of local lesions in *Nicotiana glutinosa* inoculated with either type tobacco mosaic virus or the masked strain, and kept first at 36° and then at 20°. Most were very large and appeared quickly; others were much smaller and appeared 3–4 days after the plants were placed at 20°. It seems that the virus causing the smaller lesions either lay dormant during the period at 36° or, perhaps more likely, became established in the initially infected cells but was unable to spread from them until the temperature was lowered. The virus causing the larger lesions, although unable to reach such amounts as at 20°, nevertheless readily spread from cell to cell in leaves at 36°.

The effects of varying the temperature at which plants are grown vary so much with different viruses and virus strains, and with the identity and physiological condition of the host plants, that here we can do little more than indicate their great variety, mainly to show what a wealth of research problems there are and how these promise results that should greatly help to understand the factors that influence infection, virus multiplication and inactivation, mutation, and the spread of virus from cell to cell.

4. *Inhibitors and Enhancers of Infection*

The number of infections obtained with a given inoculum does not depend only on the amount of infective virus it contains and the susceptibility of the plants inoculated, but also on other components of the inoculum. Most other components decrease infectivity and this ability is common to so many substances that it is not surprising that most biological fluids inhibit infection. The inhibitors whose identity has been determined range from the enzymes trypsin and ribonuclease, and various other proteins, through polypeptides and polysaccharides, to small molecules of which the most studied are analogues of purines and pyrimidines. The behavior of these substances has recently been reviewed in detail (Bawden, 1954), so that little more than a summary need be given here.

How the inhibitors of infection act is still uncertain, but the suggestion has already been made in Section III that ribonuclease, which is one of the most powerful inhibitors of infection, may destroy the nucleic acid when this becomes separated from the virus protein during an initial step in the infection process. Many other inhibitors that behave superficially like ribonuclease, however, have no nuclease activity, and these presumably act in some other manner or stimulate the normal cell nucleases into extra activity. Some inhibitors combine with virus particles *in vitro*, and these could act by blocking some groups on the particles that need to be free if the virus has to combine with some specific infection site in the host cells as a preliminary to multiplication. Others, however, inhibit without combining with the virus particles, and so again this blocking of essential groups can not apply to all inhibitors. The activity of most of these inhibitors of infection is determined less by the nature of the virus used than by the identity of the plant inoculated. It seems, then, that the host plant is involved, and the most that can be done to offer a general explanation (there is, of course, no reason to assume that different substances all act in the same way) is that inhibitors affect the metabolism of cells they enter so that the balance, always sensitive, between the inactivation and establishment of virus, is tipped in favor of inactivation.

Most inhibitors of infection act only when they are present in the inoculum or when they are rubbed over leaves which have recently been inoculated with viruses, possibly because they are substances with large molecules and cannot diffuse readily through leaves and reach cells other than the epidermis. Some substances with small molecular weights are not only inhibitors of infection, but also inhibitors of virus increase, and these can check virus multiplication even when applied after infection has occurred and multiplication has already started. The two most studied are thiouracil (Commoner and Mercer, 1951, 1952; Mercer *et al.*, 1953; Nichols, 1953; Bawden and Kassanis, 1954) and 8-azaguanine (Matthews, 1953a,b). Again their inhibiting ability varies with the identity of the host plant; thiouracil for example affects the multiplication of a tobacco necrosis virus in tobacco but not in French bean. The physiological state of the plant is also important and thiouracil more effectively interferes with the multiplication of tobacco mosaic virus in detached tobacco leaves floated in a nutrient solution and kept in the light, conditions that normally lead to a high virus content, than in leaves floated in water and kept dark, conditions that do not favor virus multiplication.

The inhibiting action on tobacco mosaic virus multiplication of thiouracil, like that of 2-thiocytosine and 2-thiothymine, is counteracted by uracil, whereas the action of none is counteracted by cytosine or thymine, suggesting that all three act by disturbing a process depending on uracil rather than the other bases. The action of 8-azaguanine is counteracted by guanine and adenine but not by uracil, xanthine, or thymine. The two substances, thiouracil and 8-azaguanine, affect different viruses to different extents; for example, in tobacco, thiouracil strongly inhibits the multiplication of tobacco mosaic virus, but has relatively little effect on cucumber mosaic virus, whereas 8-azaguanine affects cucumber mosaic virus but has little effect on the multiplication of tobacco mosaic virus. This specificity cannot yet be explained, but it clearly suggests that cucumber mosaic virus may have a higher ratio of guanine to uracil than has tobacco virus, or is in some way more dependent on the guanine metabolism of host cells.

When leaves infected with tobacco mosaic virus are treated with 8-azaguanine (Matthews, 1953b) or thiouracil (Jeener and Rosseels, 1953; Matthews, 1956), these substances become incorporated in the virus nucleic acid, replacing some of the guanine and uracil respectively, which provides a plausible explanation for the inhibition of multiplication by suggesting that particles containing these anomalous substances are "sterile." However, the explanation is not wholly satisfactory, because the small amount of virus formed in the presence of thiouracil under conditions that favor inhibition seems, weight for weight, not much less infective than normal virus. Also, that there are infective particles in such treated leaves is readily shown, either by removing the thiouracil or adding uracil, when the virus immediately starts to multiply. The fact that virus increase can be stopped by thiouracil applied some days after leaves are inoculated, when they already contain much infective virus, further suggests that the phenomenon has some other explanation than the formation of some noninfective particles, and seems to imply that thiouracil acts mainly by preventing the virus infecting new cells; there is no evidence to suggest whether this is because thiouracil disturbs the normal uracil metabolism of these cells so that some first step in the infection process is prevented, because thiouracil becomes incorporated in the first-formed virus nucleic acid and prevents its further development, or because of some quite other effect. That thiouracil does interfere with the host metabolism, however, is clearly evident when whole tobacco plants are treated with it, for apical growth ceases and the young leaves become acutely chlorotic. These effects, unlike inhibition of virus increase, are not counteracted by giving uracil.

Thiouracil has no therapeutic effect on plants infected with tobacco mosaic virus; its use stops or slows virus increase, leaving virus already formed unaffected. However, it can decrease the virus content of plants infected with some less stable viruses, probably not because it directly inactivates them but because their particles normally have only a short life *in vivo* and thiouracil prevents the synthesis of new ones to replace those that become inactivated. There is no example yet of a whole plant being freed from infection by its use, but the virus content of potato plants infected with potato virus Y was much decreased and cultures of tobacco callus tissues were made virus free by prolonged exposure to it (Kassanis and Tinsley, 1958).

Some constituents of inocula increase the number of lesions produced; they deserve mention because although they so facilitate the mechanical transmission of some viruses that their use has become almost routine, it is often overlooked that we know little or nothing about how they act. The most used are substances usually designated "abrasives," of which fine Carborundum and types of diatomaceous earths are most effective, able to increase infections by a factor of 100 or so. No doubt they do increase the number of wounds produced when leaves are rubbed and so increase the number of entry points for viruses, but it seems likely that they have other effects than this; for example, virus multiplication is detectable and lesions appear sooner when they are used than when they are not, suggesting that the type of injury they produce also makes conditions in the injured cells more conducive to the successful establishment of infection. The other constituent of inocula widely used to increase infectivity is phosphate, which can hardly be expected to increase the number of wounds and so must act in some other way. It is more effective with some hosts than others and its action also varies with the age of leaves (Yarwood, 1952a).

When inocula contain substances that injure leaves, washing the inoculated leaves usually increases the number of infections, but when they do not, washing leaves with water within 30 minutes of inoculation decreases the number (Yarwood, 1952b, 1955; Bawden and Harrison, 1955). Whether this means that after this time all the virus particles are firmly attached to some component of the host cells, or that the cells are no longer permeable to virus particles because the wounds produced at the time of inoculation have healed, cannot be concluded.

5. *The Presence of Other Viruses*

Of the many ways in which the presence of one virus in a plant can affect the multiplication of another, the most studied has been the ability of related strains of a given virus to interfere with each other's multiplication. Since Thung (1931) working with tobacco mosaic virus, and Salaman (1933) with potato virus X, showed that plants infected with an avirulent strain resisted infection with virulent ones, this phenomenon has been found to be fairly general and "plant-protection" tests have been widely used to relate symptomatologically different viruses as related strains. In general, viruses that share antigens protect plants against one another, and the more closely related they are serologically, the greater is the degree of protection afforded. Tests of relationships are usually made by inoculating the second ("challenge") virus to plants or leaves already fully infected with the other, and noting whether the second virus produces any additional effect, but related strains also mutually interfere when inoculated simultaneously to the same tissues. The result of such mixed infections is then a compromise, both in symptoms shown and in virus content, between what would have been achieved by either strain alone. Identifying clinically distinct viruses as related strains is usually not difficult from such tests because pairs of unrelated viruses mostly multiply independently of one another, each producing its characteristic effects and reaching amounts comparable to when it is present alone; or they interact to produce more severe symptoms than either produces alone, when one or the other may also reach a higher concentration than when on its own.

Past explanations of these interactions between viruses have been mainly based on the idea that strains of one virus compete with one another, either for a limited number of specific infection sites in host cells or for a limited amount of some essential substance, whereas unrelated viruses do not. When one strain is already established, all the infection sites are assumed to be occupied, or all the raw materials for that type of virus used, leaving none for a second strain, although viruses that multiply at different sites or use other raw materials will still be able to multiply. Best (1954) has suggested that a better explanation for the plant-protection and interference phenomena is that, while multiplying in the same cell, strains of one virus exchange genetic determinants. On this theory, a virulent strain entering a cell already infected with an avirulent one would exchange some determinants with it and so lose the specific combination of genetic characters conferring virulence. The idea that an essential step in the infection of cells is the break down of the infecting particle into nucleic acid and protein suggests yet another explanation. As fully infected cells contain virus particles, which are nucleoprotein and are stable over periods much longer than is needed for infection to occur in healthy cells, it seems that, if healthy cells contain a system able to separate the two components of infecting particles, this system is put out of action when infection is established and virus starts to multiply. This argument takes us several links along a chain of hypotheses with no supporting experimental facts, but the failure of a second strain to become established where one is already multiplying could mean that the second remains as nucleoprotein particles unable to take an initial step in infection. The explanation is fanciful and necessitates the conclusion that unrelated viruses are separated into their nucleic acid and protein by different host-cell systems, which is neither more nor less plausible than the idea that unrelated viruses multiply at different sites, and, indeed, could be saying much the same thing in different words. The genetic explanation has the attraction of being by far the simplest and of invoking no specific host systems or constituents; it demands only specific interactions between related strains, interactions of a type generally accepted as being taxonomically significant, for ease of crossing (genetic recombination) in organisms is one of the main criteria of near relationship. The idea that infection removes from host cells systems that disrupt virus particles into protein and nucleic acid is, perhaps, more worth mentioning as a necessary corollary to the idea that this disruption occurs as an initial step in infection, than as an attempt to explain the ability of one strain of a virus to interfere with the multiplication of another.

It is, of course, easier to show that avirulent strains protect plants against virulent ones than to show the reverse effect, but whenever the interference between strains has been studied in detail it has been found to be reciprocal. That is to say, if an avirulent strain protects plants against a virulent one, plants infected with the virulent one will also resist infection by the avirulent. There are pairs of serologically unrelated viruses, however, of which one will prevent plants from becoming infected with the other, but the protection is not reciprocal. Tobacco plants infected with tobacco etch virus, for example, resist infection with potato virus Y whereas the etch virus invades plants already infected with potato virus Y as readily as it does normal plants (Bawden and

Kassanis, 1945). The content of virus Y steadily declines as tissues become invaded by the etch virus, until after 2 weeks or so none is detectable. It seems unlikely that the etch virus directly inactivates virus Y, because the two viruses have very similar stabilities *in vitro;* more probably infection with etch virus prevents the synthesis of virus Y, which is normally maintained at a more or less constant amount only because continuing synthesis balances what is being steadily inactivated. There is no evidence to suggest what kind of change the etch virus produces in cells that prohibits the multiplication of virus Y.

Almost the opposite effect to that of severe etch virus on potato virus Y has been noted with some other pairs of viruses. The increase in content of dodder latent mosaic virus when "recovered" plants are infected with tobacco mosaic virus (Bennett, 1949) has been mentioned earlier in this chapter, and simultaneous infection with potato viruses X and Y results in leaves containing several times as much potato virus X as in comparable leaves infected with this virus alone (Rochow and Ross, 1955). Seemingly, one virus of the pair removes or affects some factor that normally would limit the amount of the other, but whether synthesis of some essential component of the other is increased, or some inactivating mechanism is decreased, is unknown.

V. Conclusion

Our incomplete story of virus multiplication can be summarized simply. Soon after inoculation to a host plant, virus particles change their state and become unstable, possibly because their protein and nucleic acid become separated. The chances of inactivation during this period are considerable and vary greatly with changes in the physiological state of the host. Survival apparently depends on the nucleic acid becoming incorporated in host-cell mechanisms that control the metabolism of the cell. Consequently, synthesis takes new directions and within a few hours virus nucleic acid and protein are formed, first as separate entities, which then combine into complete virus particles by an assembly process essentially different from multiplication. Indeed the word multiplication seems applicable only to the formation of the nucleic acid, for the requisite protein can occur in cells invaded only by nucleic acid and where there is no virus protein either to multiply or to act as a pattern for its own synthesis. Seemingly, the structure of the nucleic acid not only allows it to be self-replicating but also to determine the type of protein made. While the nucleic acid is increasing, opportunities arise for it to change its structure, either by faulty copying (mutation) or by exchanging parts of its structure with other, related, nucleic acid.

Whether the protein plays any role other than protecting the nucleic acid is unknown, but this is vital because the nucleic acid is readily inactivated by exposure to many conditions that are harmless to completed virus particles. Virus formation in any cell increases to a limit that varies with different viruses and with different physiological states of the host cells; it seems to be a continuing rather than a once-and-for-all process, with the content of completed particles at any one time depending on the balance between their formation and breakdown. The virus content of leaves continues to increase for many days after they are inoculated with a virus that becomes systemic, because an increasing number of cells become infected by virus spreading from earlier infected cells in which it has already increased. In each newly infected cell, the same sequence of events is probably repeated, but with less uncertainty about the result, because when virus from the inoculum becomes inactivated in the first infected cells, there is none to replace it, whereas the supply for cells invaded later is almost unlimited. However, there are conditions in which cells can be infected but the virus in them seems unable to invade or become established in its neighbors.

The study of plant viruses is still much in the stage of uncovering phenomena rather than of explaining them, and to tell even this incomplete story entails much speculation and extrapolation to an almost unreasonable extent from results with bacteriophages and tobacco mosaic virus. These are favorable experimental material and will doubtless continue to provide much new information, but other viruses urgently need study to know whether these most studied are representative or exceptional. I have tried to show that some features of other viruses can be fitted into the general story, but these others are sadly few and information, even on these few, is woefully small. I have also tried to distinguish between facts and speculation, but if I have not always succeeded I hope the obscure statements will be treated as speculations. This way no harm is done, for speculations and theories serve a useful purpose only as stimulators of research and as "Aunt Sallies" to be shot at, whereas to assume that more is known than is, only stultifies research and perpetuates error. Ideas about virus multiplication are changing almost continuously, and virus research is a rapidly developing subject. Doubtless, much in this chapter will soon be out of date, for I can present only an *interim* report. It will serve its purpose if the obvious gaps in knowledge indicate what significant discoveries there are yet to be made and attract more workers to a subject that promises to yield results that will be important and significant, not only in plant pathology, but in most branches of biology.

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CHAPTER 4

Reproduction of Bacteria, Actinomycetes, and Fungi

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When a pathogenic organism has reached an appropriate host it must, in order to be successful, establish itself in or on that host. Many plant pathogens remain restricted to a small area of localized infection, as with many fungi causing leaf spots. Some, such as the small highly specialized group of the powdery mildews, are almost entirely external to the host, spreading over the surface and putting haustoria into the epidermal cells, and others spread throughout the host and become systemic in it. Whatever the final distribution of the parasite on or in the host may be, active multiplication of the invading cells is an essential stage and may be considered to be a form of vegetative reproduction. When the host has either succumbed to the attack by the parasite or has died or become dormant from other causes, the parasite must either enter into a resting phase or escape from the original host and migrate to new ones. Reproductive stages fulfill both these requirements. Many pathogens produce both resting cells or organs and other structures adapted for dispersal. Where the life cycle of a pathogen includes a sexual stage, the increased chance of the emergence of new strains, through the rearrangement of nuclear material, offers a third advantage in the struggle for survival. A well-known example of this is the production of hybrid strains of *Puccinia graminis* which may have a slightly different host range from that of the parent strains and may, therefore, be able to invade wheat varieties immune to the latter.

Reproduction is thus of primary importance to plant pathogens as a means of securing their survival and spread and of increasing their variability and hence their power of exploiting new situations. Many different types of reproduction are found among plant parasites and a close correlation may usually be traced between the life cycle of a parasite and the type of disease it causes.

I. Bacteria

The bacteria show a very limited range of methods of reproduction. All of them multiply by simple fission of the vegetative cell. This takes place rapidly and repeatedly under favorable conditions and is then a most efficient means of multiplication and potential spread of the organism. Endospores are produced by the group Bacillaceae and by a few other forms, but these are sedentary, resistant spores rather than agents of mutliplication and spread. Most plant pathogenic bacteria, moreover, are nonsporing rods. Sexual reproduction in bacteria is still a matter of controversy and the part it plays, if any, in the life cycle of a bacterial plant parasite is obscure.

Parasitic bacteria enter the plant either through wounds or through such natural openings as stomata or lenticels. They have no power of penetrating the uninjured cuticle. Entry is, therefore, a passive process. Both parasites and saprophytes enter injured plant tissues but only those able to multiply and survive within the living parts of the plant become established. Here a rapid increase in the number of cells is important. Such multiplication is also essential for escape from a dead or dying host and in many bacterial diseases of plants the final phase is the exudation of slimy masses of bacteria from the dead tissues. Individual bacterial cells may reach new hosts by purely passive methods of distribution, such as in water splashes or by dispersal by insects. The important feature here is the rapid production of very large numbers of cells.

A. *Multiplication by Division of Individual Cells*

Multiplication of bacteria by binary fission is a simple and efficient means of cell reproduction. The speed with which it takes place varies with the species and with environmental conditions, but when the latter are favorable, the rate of increase in number of cells may be very great.

Owing to the small size of bacteria considerable doubt exists as to the exact nature of even such an apparently simple process as binary fission. The process is essentially the separation of two daughter cells formed by the development of a wall across the original parent cell.

Since each new cell must carry the characters necessary for independent existence, some division of the nucelar material of the parent cell is an essential preliminary to binary fission. Neither the exact form nor the method of division of a bacterial nucleus is as yet fully determined. Bissett (1956) believes that the most common form of a bacterial nucleus is rod shaped and that this rod divides transversely at a central constriction. He suggests that the apparently simple rod may consist of a number of distinct nuclei, which are themselves rod shaped and arranged parallel to one another. Whether or not this is a true picture of the bacterial nucleus, it is clear that some such division of nuclear material must take place during cell division.

Most observers agree that the next step in fission is the formation by the plasma membrane of a median septum dividing the protoplast into two parts (Fig. 1a). It is now also generally agreed that after the formation of this septum, the lateral cell wall grows inward in the same plane separating the two daughter protoplasts (Fig. 1, b–d). This ingrowth of the cell wall is clearly shown in electron micrographs of thin sections of *Bacillus cereus* by Chapman and Hillier (1953).

After the formation of the cross wall, the daughter cells may separate at once or the wall may be incomplete so that they remain attached by fine protoplasmic strands. Actively dividing bacteria may remain connected in chains or clusters for some time before final separation of the cells is achieved by the snapping of the connecting strands.

Both multiplication and the provision of dispersal units of plant parasitic bacteria are achieved by the comparatively simple process of binary fission. Large numbers of bacterial cells in slimy masses are seen within the tissues of infected plants in such diseases as the yellow disease of hyacinth, caused by *Xanthomonas hyacinthi*. In wet weather similar masses are seen to ooze from lesions on the shoots of various trees, as with the walnut attacked by *Xanthomonas juglandis*, or the pear attacked by fire blight (*Bacterium amylovorum* syn. *Erwinia amylovora*). These surface exudations of bacteria are readily dispersed by various agencies, such as raindrops or insects. Thus, in these examples, fission leads to spread of the pathogen within the host plant and to dispersal beyond it.

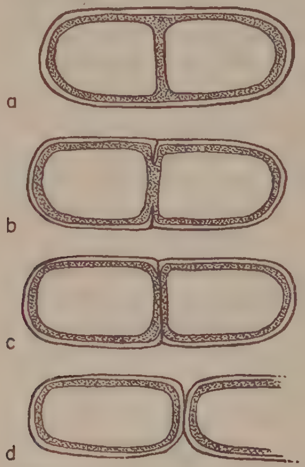

FIG. 1. Binary fission in bacteria: (a) Cell membrane has formed a septum across rod-shaped cell. (b) Wall beginning to grow inward along plane of septum. (c) Cross-wall completed. (d) Daughter cells separating.

In many bacterial diseases of plants, such as various leaf spots, or the scab diseases of gladiolus (due to *Bacterium marginatum*), such conspicuous masses of bacterial cells are seldom seen and the injury is usually localized. Nevertheless, the progress and spread of these diseases also are dependent on multiplication of cells by fission even though the process is slower and results in the formation of fewer cells than with the more spectacular diseases mentioned above.

B. *Spore Formation*

Endospores are produced under particular circumstances by a number of rod-shaped bacteria of the family Bacillaceae. No important bacterial pathogens of plants have been shown to produce spores but it is by no means excluded that more detailed investigations of their life cycles might demonstrate occasional spore production. The great resistance of bacterial spores to desiccation and toxic substances is obviously of survival value. Dowson (1949) includes the spore-forming *Bacillus mycoides* and *B. megaterium* in his account of bacteria causing plant disease. These species frequently occur on isolation plates prepared from diseased roots or other material contaminated by soil, but it is not clear whether they cause disease. Their power of survival in the soil must be enormously increased by the ability to form spores.

Endospores are formed by the condensation of the cytoplasm of the vegetative cell, either through the aggregation of granules which migrate to the pole of the cell or, more probably, by condensation of the contents of a clear spore primordium surrounded by a membrane (Knaysi, 1942). The mature spore is surrounded by a definite wall which is thought to consist of three distinct layers. Analysis of the spores and vegetative cells of *B. mycoides* shows that water content, protein, and ash are similar in both (Virtanen and Pulkki, 1933). Earlier investigators assumed that water content of the spores is lower than that of the vegetative cell and that this in part accounts for the resistant properties of the former. It has been suggested that this resistance is due to the relatively low water content of the spore and to the inactivation of the enzyme systems (Dubos, 1949).

Spore formation is usually considered to be stimulated by exhaustion of nutrients, but other conditions such as aeration may also be important. Sudden changes to less favorable conditions do not usually result in spore formation. Spore-forming species may give rise to permanently nonspore-producing mutants. More exact information on the conditions leading to spore formation is desirable together with detailed investigations of the life cycles of plant pathogenic bacteria to determine whether some of these occasionally produce spores.

C. *Possible Sexual Reproduction in Bacteria and its Significance*

Evidence for a sexual process in the life cycle of bacteria is provided by direct observation of apparently conjugating cells and the subsequent production of microcysts or other small cells (gonidia), the formation of bodies strikingly unlike those of the normal vegetative cell, the interpretation of dark staining chromosome-like bodies during cell division, and the recombination of genetic characters in individuals from mixed cultures. Very little work has been done recently on the possible sexual stages of bacteria parasitic on plants, but one of the earliest claims that sexuality exists among bacteria was that of Stoughton (1930, 1932) who worked with *Bacterium malvacearum* (*Xanthomonas malvacearum*), the cause of black arm disease of cotton.

The nature of the bacterial nucleus has long been a matter of controversy. Technical advances, such as improved staining techniques and the introduction of the electron microscope, have provided evidence in support of the view that the bacterial nucleus is essentially similar in structure and behavior to that of other organisms. Chromosome-like bodies have been seen by some observers (Robinow, 1956; De Lamater, 1956) and it has been claimed that the arrangement of these at certain stages in the life cycle indicates that a typical reduction division takes place (De Lamater, 1956).

The most convincing evidence for some sort of sexual process in bacteria is obtained from the study of mutant strains and strongly suggests that segregation of characters, such as would result from a reduction division, takes place in many bacteria.

While much of the evidence brought forward to prove the presence of a sexual process in bacteria is not conclusive, it is becoming increasingly clear that sexual phases are not uncommon among bacteria. If sexual reproduction does take place, it is clear that the formation of hybrid or mutant strains is of great importance among pathogenic species. The ability to produce new strains confers upon the bacterial population the ability to colonize new substrata and to survive under new and hitherto unfavorable conditions.

D. *Conditions Influencing Reproduction of Bacteria Pathogenic on Plants*

Multiplication by fission is thus the most frequent, or even perhaps the only, form of reproduction of plant pathogenic bacteria. This process is obviously dependent on all conditions being favorable, since the vegetative cell is highly vulnerable to the effect of harmful factors. The newly divided cell, with a thin end wall, is probably even more susceptible to damage than is a mature cell. The environmental factors most likely to influence cell multiplication within the host are food supply, temperature, water content, H-ion concentration, and aeration, while the formation of bacterial slime at the surface of the host is particularly dependent upon the humidity of the air. Thus, the multiplication and dispersal of bacterial plant pathogens are dependent upon the weather. The absence of resistant spores or other resistant structures accounts for the close correlation between the incidence of bacterial diseases of plants and the weather. It is perhaps not too much to claim that this high degree of dependence of reproduction on external conditions may account for the fact that the bacteria are, in general, less successful than the fungi as plant parasites.

II. ACTINOMYCETES

The actinomycetes have been variously classified among the higher bacteria, in the Fungi Imperfecti, or as a separate group intermediate between bacteria and fungi. Waksman (1950) treats them as a separate group, the Actinomycetales, distinct from the Eubacteriales and divided into three families; (1) Mycobacteriaceae, in which the mycelium is rudimentary or absent, (2) Actinomycetaceae, in which the vegetative mycelium readily fragments into bacillary or coccoid segments, and (3) Streptomycetaceae, in which the vegetative mycelium does not normally fragment and in which true spores are produced. Two genera, *Mycobacterium* and *Streptomyces*, include species causing plant disease. *Mycobacterium phlei* causes a disease of timothy grass and *M. rubiacearum* induces the production of small nodules on the leaves of certain members of the Rubiaceae. The latter species is usually considered to be a controlled parasite or symbiont, since its capacity to fix atmospheric nitrogen within the leaf nodules undoubtedly benefits the host. Most plant pathologists consider *Mycobacterium* with the bacteria. The filamentous *Streptomyces* includes *S. scabies*, the cause of common scab of potato, and some other species causing plant diseases of less importance. It is often included in the fungi by plant pathologists.

A. *Vegetative Multiplication*

The individuals of species of *Mycobacterium* are more or less elongated rods and have a characteristic beaded appearance. They are usually considered to consist of a short, probably septate, filament. As these grow in length they fragment by a process similar to the binary fission of the true bacteria. Multiplication of individuals and the provision of dispersal units is, thus, similar in *Mycobacterium* and the true bacteria. The fragmentation of the longer branched filaments of the Actinomycetaceae also serves the same purpose of an increase in the number of individuals and the production of units suitable for dispersal and consequent spread of the organism. Again, as with the bacteria, this process is dependent upon a favorable environment.

Fragmentation of the filaments of *Streptomyces* seldom takes place. It is not definitely established whether the filaments are septate or not, but in either case growth in length leads to an increase in bulk of the vegetative thallus and, thus, to further penetration of the plant host. In that sense, growth may be considered to be a form of reproduction, but *Streptomyces*, unlike *Mycobacterium* and the true bacteria, does not produce vegetative dispersal units. The dispersal unit here is a true spore, as in most fungi. With the development of these special asexual reproductive units, increase in bulk of the thallus and the provision of dispersal units are no longer achieved by one and the same comparatively simple process of cell division.

B. *Spore Formation*

The spores, or so-called conidia, of *Streptomyces* are produced in chains on aerial hyphae. On different species these chains may be branched, straight or spiral (in clockwise or anticlockwise direction), single or in tufts. All are similarly formed by the rounding-off of the protoplasm into spherical masses between which cross walls develop by ingrowth of the lateral wall. The cells thus formed round off and finally separate as distinct spores. Sporulation proceeds from the tip of the hypha toward the base and, thus, these spores are oidia or arthrospores rather than true conidia. In some Actinomycetes, notably species of *Nocardia,* thick walled chlamydospores are formed in the vegetative mycelium. The spores of *Micromonospora,* which are formed singly on short side branches, have also been interpreted as chlamydospores by some observers (Waksman, 1950).

Klieneberger-Nobel (1947) claims that sporulation in *Streptomyces* is preceded by a fusion of sexual branches, but while fusions of hyphae have been observed by other investigators, it is not clear whether these are sexual fusions or whether they are anastomoses between vegetative hyphae of the type common among true fungi. Whether or not these fusions are true sexual ones, they may well lead to a redistribution of nuclear material and a consequent increase in variability.

III. Fungi

The life cycle of a plant parasitic fungus may be divided into a number of biological phases. (1) The first phase is that of penetration of the host. Reproduction plays no part in actual penetration. (2) When penetration of the host by an infection hypha or haustorium has been achieved, the second phase of establishment within or on the host begins. This involves vegetative multiplication of hyphae which continues until the host is destroyed or fungal growth is checked by host resistance or other adverse environmental conditions. (3) Before this stage is reached, a third phase of rapid spread of the parasite to new hosts usually begins. Reproduction plays a major part here by the formation of spores or other infection units, called propagules by Garrett (See Chapter 2 of Volume III). The exploitation of the original host and the spread of the parasite to new ones continues as long as conditions are favorable to growth and reproduction of the fungus, but when, either as a result of death of the host and exhaustion of food supply or of seasonal changes, such as the approach of winter cold or summer drought, the environment becomes less favorable, the pathogen enters a phase of resistance to the unfavorable conditions. (4) Here reproduction is again of primary importance and while some fungi are able to survive throughout the year in a vegetative or asexual state, the majority produce resting bodies protected by thick walls or other devices. The most frequent form of resistant body is a resting spore, which may often be produced as the result of a sexual process. Thick walled vegetative bodies, such as sclerotia may play a similar part in resistance to temporary unfavorable conditions. (5) With the return of favorable conditions reproduction is again the means by which new infection units are produced in sufficient numbers to enable the parasite to become established on suitable hosts once more. Thus, the success of nearly all fungi parasitic on plants is dependent at some stage of the biological life cycle on some form of reproduction. Reproductive processes of fungi are much more varied than those of bacteria and Actinomycetes and this may in large part account for the relatively better success of the group in parasitizing plants.

A. *Vegetative Reproduction*

1. *Growth of Hyphae*

The vegetative thallus of most fungi is a mycelium composed of branched filamentous hyphae, which enables the fungus to spread within the host. Many fungal parasites ultimately ramify throughout the intercellular spaces of a whole plant, and are then said to be systemic. Others penetrate and destroy the cells of the host plant, while yet others are unable to spread far beyond the point of penetration and remain as localized infections. Hyphae grow from the tip area but branching (including the formation of haustoria), thickening of the walls, vacuolation of the protoplast, the development of pigment, and other changes may take place in older parts of the hypha. Such a vegetative increase in bulk is not usually considered to be a form of reproduction, but it performs the same function as binary fission in the bacteria. Hyphal growth and branching is a more efficient method of spread within the host than is shown by the bacteria, since the hyphae actively advance and push their way between or into the host cells, but it is inefficient in providing propagules.

2. *Budding, Formation of Oidia*

A closer parallel to the bacteria is seen among the yeasts. True yeasts do not produce stable hyphal filaments but exist in the form of a sprout mycelium, that is as a mass of single cells. They multiply either by binary fission, as in the bacteria, (e.g., species of *Schizosaccharomyces*) or by budding (Fig. 2, a, b) as with the majority of yeasts, including the genus *Saccharomyces*, species of which are used in alcoholic fermentation. In a budding yeast, under favorable conditions of nutrition and other factors, the cell puts out one or more protrusions or buds which grow in size and finally round off and separate from the parent cell. In an actively growing colony of either a fission or a budding yeast, the final separation of the new cells may be delayed and short chains of cells may

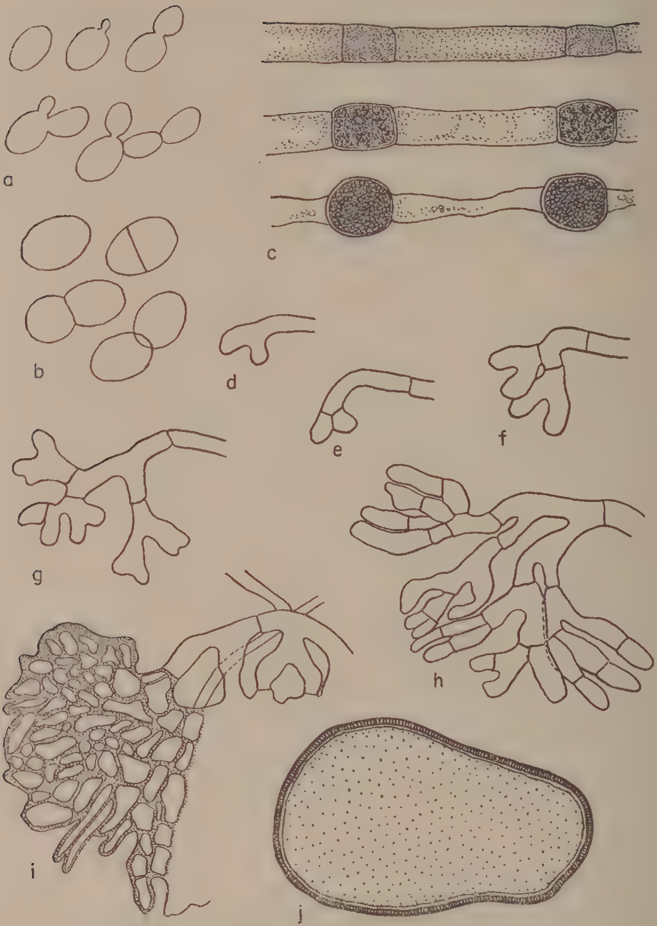

FIG. 2. Vegetative reproduction in fungi: (a) Budding cells of *Saccharomyces carlsbergensis.* (b) Cells of *Schizosaccharomyces octosporus* dividing by fission. (c) *Mucor racemosus,* formation of chlamydospores. (d–i) Stages in formation of sclerotium of *Botrytis allii* by repeated dichotomy of original hypha, followed by anastomosis and adhesion of branches and thickening of cell walls. (j) Transverse section, mature sclerotium of *B. allii,* showing extent of outer rind of thick walled cells. Magnification: a, b, × 800; c, × 400; d–i, × 500; j, × 20. d–j after B. B. Townsend and H. J. Willetts, *Brit. Mycol. Soc. Trans.* **37,** 1954.

be formed temporarily. The true yeasts are not pathogenic to plants, but certain yeast-like fungi, the so-called fungi of stigmatomycosis, e.g., *Nematospora coryli*, which readily form a sprout mycelium, cause diseases of cotton and other plants. Many plant parasites, such as *Taphrina deformans* and certain smuts, readily produce a sprout mycelium or form oidia in culture or under certain conditions in nature. The so-called "mirror yeasts," *Sporobolomyces* and *Bullera*, are common epiphytes on various plants but are not known to cause disease. On the whole, the part played by an oidial or yeast-like stage in the life cycle of fungal parasites is negligible.

3. *Chlamydospores*

As a fungal hypha grows at the tip, the older parts tend to become empty of contents and, with normally aseptate species, are cut off by septa from the living parts. In some fungi, before this stage is reached, the contents collect in certain cells of the hypha, in terminal cells of short branches, or in certain parts of an aseptate hypha, and become oily and surrounded by a more or less thick wall. Such a body is known as a chlamydospore (Fig. 2c). It is generally assumed, but without any conclusive evidence, that the formation of chlamydospores is induced by the onset of unfavorable conditions, notably by a fall in concentration of available food materials. In particular species, chlamydospores are induced by adverse nutritional conditions such as a high concentration of sugar (e.g., species of *Mucor*), a low carbon-nitrogen ratio (*Fusarium oxysporum;* Carlile, 1956), or a general reduction in available food supplies (*Saprolegnia* spp.), and by the presence of antagonistic bacteria (*Fusarium* spp.; Venkat Ram, 1952; Park, 1954). Chlamydospores are often of a resistant nature, owing to their thick walls and high density. Among plant parasites, species of *Fusarium* are well known to form chlamydospores readily and it is likely that these play a part in the survival of these fungi in the soil or on dead host plants.

4. *Sclerotia*

Resistant bodies of a more complex nature are formed vegetatively by many plant pathogenic fungi, particularly those which are soil-borne. These bodies, known as sclerotia, consist of aggregations of hyphae and are formed in various ways by the repeated branching and anastomosis of the constituent hyphae (Townsend and Willetts, 1954). The individual cells of these hyphae often round off so that in section the sclerotium looks like a true tissue. Some sclerotia, e.g., those of *Rhizoctonia solani*, are more or less uniform in structure throughout, all the constituent cells being similar and thick walled. This type may be better considered as a mass of chlamydospores than as a true sclerotium. More frequently, sclerotia show an outer protective zone of thick walled cells, usually dark colored, and an inner mass of cells with thinner hyaline walls and dense contents (Fig. 2, d–j). Reserve foods, including oils and glycogen, are accumulated in sclerotia so that the structure is well adapted for survival over a period of adverse conditions. Some sclerotia germinate by putting out masses of vegetative hyphae but many produce specialized stromata on or in which the sexual spores are borne (e.g., the ergot, *Claviceps purpurea*, or various species of *Sclerotinia*). Fungi which produce sclerotia are often able to survive for long periods in the absence of the host plant and are often (as with *Phymatotrichum*, the cause of cotton root rot, difficult to eradicate from the soil.

B. *Asexual Reproduction*

The most usual method of spread of plant pathogenic fungi during periods of favorable environmental conditions is by large numbers of asexual spores. In the large group of the Fungi Imperfecti no other form of spore is known and in many Phycomycetes and Ascomycetes asexual spores predominate. These spores are usually small, contain no great accumulation of reserve foods, have comparatively thin walls and are produced in large numbers. They are, therefore, a relatively economical method of reproduction and, under conditions favoring their formation, dispersal, and germination, are a highly efficient means of spread of the fungus to new hosts.

Asexual spores may be motile zoospores which are formed in zoosporangia by many lower fungi; they may be nonmotile but formed in sporangia, as with many members of the Mucorales; or they may be produced directly on the mycelium or on specialized hyphae or conidiophores, as in many higher fungi.

1. *Zoospores*

Zoospores are produced only by certain lower fungi and are typical of these members of this group which occupy aquatic or semiaquatic habitats. They are formed in special sac-like cells, the zoosporangia. In the unicellular holocarpic chytrids, the zoosporangia are usually flask shaped and are formed from the whole unicellular thallus which is used up in the process (Fig. 3, d–f). In *Synchytrium endobioticum* (causing black wart disease of the potato), the whole thallus is extruded into a vesicle, or prosorus in which a group or sorus of zoosporangia is formed (Fig. 3, a–c). The plasmodium of *Plasmodiophora brassicae* (causing club root of crucifer) which fills the host cells, eventually forms a mass of spores, each of which later sets free a zoospore into the soil. In the mycelial groups, of which the Oomycetes are the most important, the zoosporangia are intercalary or terminal in the simpler species and terminal on simple or branched specialized hyphae or sporangiophores in the more advanced ones (Fig. 3, g–m). They are usually cylindrical or globose and become more clearly differentiated from the vegetative hyphae in the higher Oomycetes. The young zoosporangium has relatively dense contents and, in hyphal species, becomes cut off from the parent hypha by a septum. Fissures develop in the cytoplasm of the zoosporangium cutting out uninucleate polygonal blocks which round off and develop cilia. The differentiation of the zoospores takes place inside the zoosporangium in most species, but in *Pythium* the contents of the latter are extruded into a vesicle where the zoospores are formed. A similar vesicle is sometimes formed by zoosporangia of some species of *Phytophthora,* but the zoospores are usually formed within the sporangium before extrusion into the vesicle. The zoospores are shed by the opening of a special apical pore in the wall of the zoosporangium (as in species of *Saprolegnia*), by the removal of a small lid or operculum (as in some chytrids), or most commonly by the bursting of the wall of the zoosporangium or of the vesicle arising from it. On release, the zoospores are naked globules of protoplasm, lacking a cell wall, uninucleate and possessing one or two cilia or flagella. The shape of the zoospore (pyriform or reniform) and the number and arrangement of the flagella are of taxonomic importance. Zoospores of the chytrids, Blastocladiales, and Monoblepharidales are uniflagellate, those of the Plasmodiophorales are biflagellate but of unequal length, one being very short, and those of the Oomycetes have two or more less equal flagella. Zoospores usually swim actively for some time after they are shed and then come to rest, withdraw their flagella, and become surrounded by a cell wall. Germination occurs usually by the production of a germ tube. In the water molds (Saprolegniales) the story is more complicated. Many species exhibit the phenomenon of diplanetism, that is the zoospores have two motile phases separated by a period of encystment. The shape of the zoospores in the first phase is pyriform but in the second phase it is reniform. In the higher Oomycetes there is a tendency, as seen in *Pythium* and *Phytophthora,* for the zoosporangium to fail to produce zoospores under relatively dry conditions and to germinate by a germ tube, thus functioning as a conidium. This tendency to omit the motile phase increases in the Peronosporaceae until in *Peronospora* and *Bremia* zoospores are unknown.

In many Phycomycetes germination of the sexually produced resting spore occurs by the production of zoospores, but in the more advanced species by a germ tube. This decrease in dependence on the presence

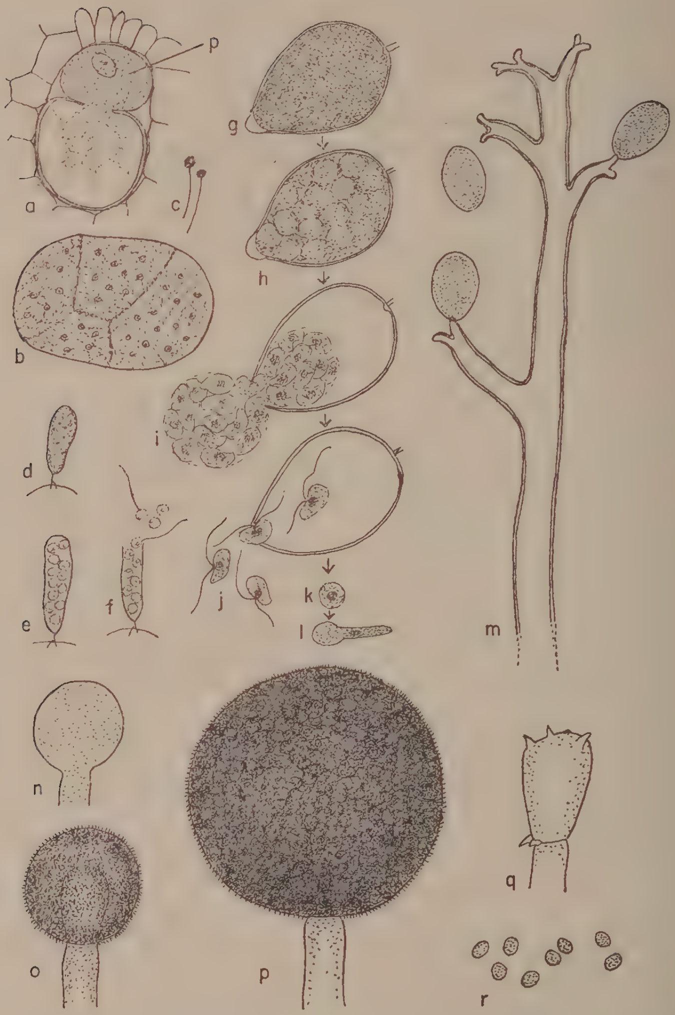

FIG. 3. Asexual reproduction of representative Phycomycetes: (a–c) *Synchytrium endobioticum* (after K. M. Curtis, *Phil. Trans. Roy. Soc. London* **B210,** 1921). (a) Developing prosorus (p) in enlarged host cell. (b) Sorus of sporangia with developing zoospores. (c) Uniflagellate zoospores. (d–f) Stages in production of zoospores of *Chytridium cocconeides,* parasitic on *Cocconeis pedicularis* (after H. M. Canter, *Brit. Mycol. Soc. Trans.* **31,** 1947). (g–l) *Phytophthora cactorum* (after E. M. Blackwell, *Brit. Mycol. Soc. Trans.* **26,** 1943). (g) Young sporangium, contents not yet differentiated. (h) Slightly older sporangium, contents partly differentiated. (i, j) Escaping biflagellate zoospores. (k) Encysted zoospore. (l) Germinating zoospore. (m) *Plasmopara nivea* on *Aegopodium podagraria,*

of free water for the dispersal of reproductive units is obviously of great advantage to fungi parasitic on terrestrial plants. Zoospores are an efficient means of reaching new host plants or other suitable substrata in an aquatic habitat or in very wet soil, but a terrestrial fungus or one parasitic on terrestrial plants is exposed to considerable hazards if its life cycle contains a motile phase.

2. *Sporangiospores*

Nonmotile spores produced in sporangia are comparatively rare among fungi and are characteristic of the simpler genera of the Mucorales such as *Mucor*, *Rhizopus*, and *Absidia*. This group does not contain any plant pathogens of major importance, although *Rhizopus nigricans* and perhaps some other species attacks overripe fruits, bulbs, and other semimoribund or dormant plant parts.

The sporangia of *R. nigricans* are produced terminally on stout unbranched or sparsely branched sporangiophores, usually arising in tufts. The sporangiophore grows upward, elongation taking place just behind the tip. The tip swells up to give a spherical sac in which the cytoplasm becomes rather dense. When this young sporangium has attained full size, a wall is laid down in such a way as to give a more or less thimble-shaped structure, the columella, projecting into the cavity of the sporangium (Fig. 3, n–r). Fissures develop in the sporangium and cut off small polygonal uninucleate blocks of cytoplasm. These round off, secrete a cell wall, and become darkly pigmented. They are finally shed by the rupture of the sporangial wall and are readily dispersed by air currents. The whole process of spore formation is very rapid and may be repeated many times so that it is a very efficient means of multiplication and spread of the fungus under conditions favoring growth.

Some members of the Mucorales produce more numerous smaller sporangia (sporangioles), each containing only a few spores, some produce both multispored sporangia and sporangioles, and a few produce their spores singly and externally, often on branched sporangiophores or on the swollen apices of these.

3. *Conidia*

Asexual reproduction reaches its highest level of efficiency among the higher fungi, particularly among the Ascomycetes and Fungi Imperfecti.

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branched sporangiophore bearing zoosporangia. (n–r) *Mucor spinosus*. (n) Young sporangium, contents undifferentiated. (o) Older sporangium, columella just visible. (p) Mature sporangium containing numerous dark sporangiospores. (g) Dehisced sporangium, showing spiny columella (characteristic of this species) and remains of torn sporangium wall. (h) Sporangiospores. Magnifications: a, × 330; b–c, × 660; g–l, × 600; m–r, × 400.

Here the usual type of asexual spore is the conidium. Conidia are single cells, or small groups of cells originating as single cells, which are not enclosed in sporangia but are formed exogenously, usually on special hyphae or conidiophores, but in some species directly on the ordinary mycelium. They are readily detached from the parent hyphae and are normally formed rapidly in large numbers. Most conidia are not much more resistant to unfavorable conditions than are the vegetative hyphae, although their contents may be rather denser and their water content correspondingly lower. Many of them are dry and readily become airborne, others are slimy and are dispersed by other means, although even some originally slimy spores find their way into the air. Conidia thus provide a relatively economical and highly efficient means of reproduction and of the rapid formation of propagules. Conidia are the chief means of rapid spread of the fungi that cause many plant diseases. They tend to be formed during the growing periods of both host and pathogen, that is, usually in the summer, and, with some exceptions, cease to form with the onset of less favorable conditions.

The term "conidium" is of wide application and covers spores of varied form and produced in a number of distinct ways by fungi of widely separate groups.

Among the Phycomycetes some members of both Oomycetes and Zygomycetes produce conidia. Under rather dry conditions, the sporangia of some species of *Pythium*, *Phytophthora*, and *Plasmopara* germinate by a germ tube instead of by the normal method of germination in which zoospores are formed and released. The "sporangia" of *Bremia* and *Peronospora* never form zoospores and always germinate by means of a germ tube. They may thus be interpreted as conidia. Among the Zygomycetes, too, the evolution of the conidium from a multispored sporangium has probably taken place. It has already been pointed out (p. 131) that some species of Mucorales produce numerous small sporangia or sporangioles, each containing a small number of spores. Forms such as *Cunninghamella*, *Piptocephalis*, and *Choanephora* produce conidia externally. Such a conidium may be considered to be a single-spored sporangium and has a double cell wall in contrast to the sporangiospores of *Mucor* which have a single wall. In the remaining groups of the Zygomycetes, namely the Entomophthorales, most members of which are parasitic on insects, and the Zoopagales, members of which are predacious on amebae and other soil protozoa, conidia are the most usual form of asexual reproductive spore.

The Fungi Imperfecti and the conidial stages of other higher fungi are classified in an arbitrary manner according to whether (1) the conidia are produced freely over the mycelium, on the ordinary hyphae or on special aerial conidiophores (the Hyphomycetes), (2) in special groups (acervuli) breaking through the outer layers of host tissue (the Melanconiales), or (3) in more or less globose bodies (pycnidia) within or on the host tissue (Sphaeropsidales). A fourth group, the Mycelia Sterilia, includes those Fungi Imperfecti which are not known to form conidia or spores of any type. The conidial forms are further subdivided according to whether the spores are readily distinguishable from the hyphae (macronemae) or not (micronemae), and on such characters as color of spores and conidiophores, septation and shape of spores, form and branching of conidiophores, formation of conidia singly or in chains, etc. This arbitrary grouping puts together species in which the mode of formation of the conidia is essentially different, and separates many obviously related forms. Attempts have been made to define the different types of conidia more clearly and to devise a more natural classification based on these fundamental characters (Vuillemin, 1910; Mason, 1933; Hughes, 1953) but so far no such classification has been generally accepted.

Vuillemin recognized two types of conidium which he termed *thallospores* and *conidia vera.* Mason suggested a third type which he called *radulospores.* Thallospores or aleuriospores have been interpreted as terminal chlamydospores, although they do not usually possess the thick wall of a typical chlamydospore. They are cut off from the parent hypha by a cross wall at an early stage of development, e.g., *Trichothecium, Helminthosporium.* Conidia vera or phialospores, on the other hand, are not cut off by a transverse wall during development. These spores are produced in basipetal succession from a phialide (a variously shaped terminal part of a hypha or hyphal branch) from the apex of which the thin walled conidia are abstricted, e.g., *Aspergillus, Penicillium.* Radulospores are each borne on a little sterigma, e.g., *Botrytis.* Other terms have been used by various authors such as *arthrospores* for "conidia" formed by the segmentation of a hypha, e.g., *Streptomyces,* and *blastospores* for spores formed by the budding of "conidia" produced as lateral buds from filamentous hyphae, e.g., the human and animal pathogens *Blastomyces* and *Candida.*

Mason (1937) also stressed the importance of the distinction between those forms with *dry* spores, Xerosporae, and those with *slime* spores, Gloiosporae. This distinction, while difficult to use as a means of classification and identification, is of great importance in considering plant pathogens, since the biological difference between dry spores, which are wind dispersed, and slime spores, which are usually dispersed by other means, is correlated with a difference in the manner in which spores of these two types reach new host plants.

Further subdivisions of spore type have been made by Hughes (1953) and others. Such attempts are of great taxonomic interest but at present are of little help in the identification of conidial stages and throw no light on the parasitic ability of plant pathogens. In a consideration of the relation between reproduction and the ability of a fungus to cause disease, the exact method of spore formation is of less importance than the efficiency of the resulting spores as agents of disease spread. Conidia formed in fundamentally different ways may have a similar function and play a similar part in the establishment of the pathogenic fungi which form them. No attempt will, therefore, be made here to give a comprehensive review of the range of developmental form among conidial species, but representative plant pathogens which produce conidia will be considered.

a. *Hyphomycetes.* The simplest form of conidia are those already described (p. 124) for species of *Streptomyces* (sometimes classed among Fungi Imperfecti by plant pathologists) in which the cells of a hypha segment and round off to form chains of spores. Hughes (1953) calls these spores "arthrospores" and considers their mode of formation to be similar to that of the conidia of some Basidiomycetes and of the imperfect genus *Geotrichum.* Other investigators have interpreted them as oidia, but they differ from true oidia in germinating by a germ tube rather than by the production of a mass of yeast-like cells by budding.

A somewhat similar type of formation of conidia is seen in *Monilia* which includes the imperfect stages of the bread molds, *Neurospora* spp., and of the species of *Sclerotinia* (*Monilinia*) causing brown rots of rosaceous fruits. Certain yeast-like fungi, including some animal pathogens better transferred to *Candida*, have also been included in *Monilia.* The species of *Monilia* which are the imperfect stage of the brown rot fungi may be taken as typical of the genus. These form large numbers of conidia in branched chains closely crowded together, and forming concentric rings on the diseased fruits. The conidia are formed acropetally and differ little from the cells of the conidiophore. The wall between two adjacent cells splits into two layers and a small plug is formed between these layers and acts as a disjunctor causing the separation of the conidia (Fig. 4a).

In a number of other genera, the conidia are produced basipetally on simple or branched chains. The generic name *Oidium* (Fig. 4b) is used by plant pathologists exclusively for the imperfect stage of the Erysiphaceae or powdery mildews, although some other forms have been included in it (Hughes, 1953) from time to time. The septate mycelium of a typical powdery mildew ramifies over the surface of the host plant and puts haustoria into the epidermal cells. From this mycelium, numer-

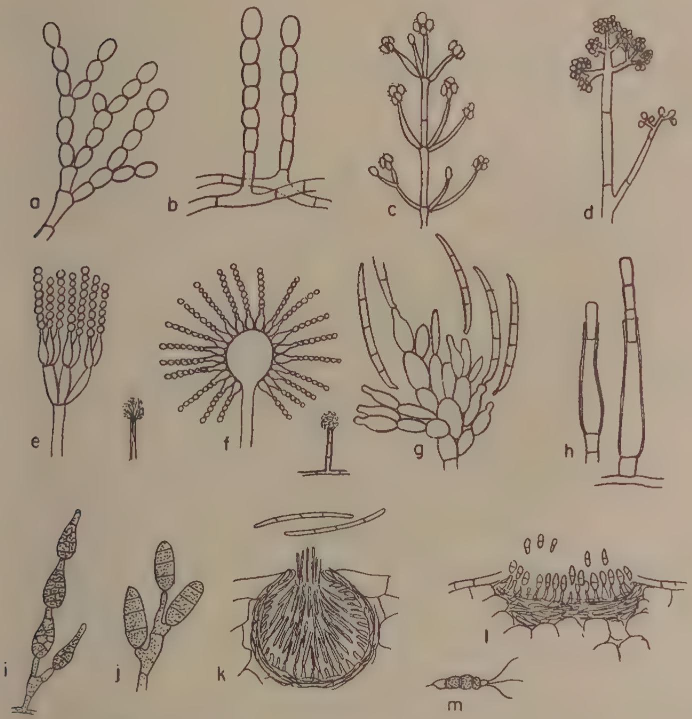

FIG. 4. Conidia: Diagrammatic sketches showing examples of different methods of formation of conidia, not drawn to uniform scale. (a) *Monilia fructigena*, branched chains of hyaline conidia. (b) *Oidium* sp., single upright chains of hyaline conidia. (c) *Verticillium albo-atrum*, conidiophore with verticillate branching, hyaline conidia terminal on branches, adhering in mucilaginous masses. (d) *Botrytis cinerea*, gray-green conidia on short sterigmata on branched conidiophore. (e) *Penicillium* sp., branched conidiophore bearing chains of blue-green conidia; small diagram of a coremium on right. (f) *Aspergillus* sp., conidia in chains arising from sterigmata projecting from swollen head of conidia; small habit diagram on right. (g) *Fusarium* sp., crescent shaped, septate spores borne on fascicles of short hyphae. (h) Endoconidia of *Thielaviopsis bascicola*. (i) *Alternaria tenuis*, dark, muriform spores in chains. (j) Dark, septate spores of *Helminthosporium* sp. (k) *Septoria apii*, pycnidium embedded in celery leaf, showing filiform conidia being cut off from short conidiophores lining cavity of pycnidium; two spores at higher magnification shown above. (1) *Marssonina pannattoniana*, acervulus breaking through epidermis of lettuce leaf, showing hyphal mat bearing short conidiophores from which two-celled spores are cut off. (m) *Pestalozzia* sp., conidium with colorless end cells, bearing appendages, and dark central cells.

ous upright chains of conidia are produced, as in Fig. 4b. The mature conidia separate readily from the chain. As is well known, the powdery appearance of these fungi is due to the numerous conidia which they produce. Many of them either do not form the ascospore stage or do so only rarely.

Monilia and *Oidium* are typical of the micronemae group of the Hyphomycetes. Other genera such as *Cladosporium*, *Ramularia*, *Cercospora*, and *Cercosporella* are borderline examples. Hughes (1953) describes the conidia of these genera as blown out ends of the hyphae. The genus *Cladosporium*, which includes both common saprophytes, such as *C. herbarum*, and important plant parasites, such as the tomato mold, *C. fulvum*, produces dark, variously branched, often clustered conidiophores from the ends of which dark colored, one-to-two celled conidia are produced singly or in chains. The wide distribution of *C. herbarum* is evidence of the efficiency of these spores as propagules. *Ramularia* includes a number of species pathogenic to a wide range of plants. The conidiophores emerge in clusters through the stomata of the host. They are short and slender and usually hyaline but are dark in some species. The conidia are hyaline and cylindrical. They commonly consist of two or more cells in a row, and frequently remain attached in short chains. *Cercospora* and *Cercosporella* are similar to *Ramularia* but produce longer spores with more numerous septations. They differ from each other in possessing dark and hyaline conidiophores, respectively. Gregory (1939) showed that the shape and degree of septation of the conidia of *Ramularia vallisambrosae* vary with the humidity of the atmosphere.

The genus *Verticillium*, which includes a number of important wilt disease fungi, is distinguished by its slender conidiophores producing whorls of branches at the ends of which small, hyaline, one-celled conidia develop singly or, more particularly in moist conditions, in small clusters (Fig. 4c).

Typical phialospores are produced by *Aspergillus* and *Penicillium* (Fig. 4c, f), large genera of mostly saprophytic habit. These are produced in enormous numbers, are light and readily detached, and are largely responsible for the almost universal occurrence of these fungi. In some of the species of *Penicillium* parasitic on bulbs, e.g., *P. corymbiferum* and *P. cyclopium*, the conidiophores tend to be long and intertwined to give composite coremia (Fig. 4e).

The common gray mold, *Botrytis cinerea*, is almost as universally distributed as *Aspergillus* and *Penicillium*, and is responsible for serious diseases of a number of unrelated plants, including many cultivated species. This fungus, too, produces numerous spores [the radulospores of Mason (1933)], each of which is produced on a small sterigma. The sterigmata are formed in clusters Fig. 4d), giving the well-known "bunch of grapes" effect characteristic of *Botrytis* species.

While the majority of conidia are hyaline and one-celled, others are more complex. Those of *Helminthosporium* and *Heterosporium*, species of which cause important diseases of cereals and other plants, are dark and consist of a row of cells (Fig. 4j). *Alternaria* produces large, dark, uniform conidia (Fig. 4i) which are usually in branched, acropetal chains. *Fusicladium*, species of which are the imperfect stages of *Venturia inaequalis* and *V. pyrina* (causing scab of apple and pear, respectively), forms a mass of subcuticular mycelium, almost stromatic in density which bears numerous crowded denticulate conidiophores which burst through the cuticle of the host and cut off numbers of dark, pyriform, one- to two-celled conidia in succession. As these are formed, the conidiophore grows up and produces another, so that conidial scars can be seen on the older conidiophores indicating the sites where conidia have been abstricted.

The large genus *Fusarium* (Fig. 4g), species of which cause many plant diseases, including destructive wilt diseases of important crop plants (e.g., cotton, banana), produces large numbers of septate, sickle-shaped macroconidia of the slime spore type. The conidiophores are variable, branched or simple, usually short and stout, single or grouped, in a mass called a sporodochium. Most species also produce smaller microconidia under certain conditions. The conidia of *Fusarium* are of the slime spore type but despite this they are frequently found in the air.

Thielaviopsis basicola, a cause of root rot in tobacco and other plants, differs from most other fungi in producing *endoconidia*, i.e., conidia formed inside the phialide (Fig. 4h).

These examples give some idea of the great variety of form of spores and conidiophores among the large group of the Hyphomycetes.

b. *Melanconiales and Sphaeropsidales.* The Melanconiales and Sphaeropsidales are smaller and more uniform groups, mainly consisting of plant pathogens. The hyphae of the Melanconiales aggregate in dense masses at or near the surface of the host and produce numerous short crowded conidiophores from the apices of which the conidia are produced. The expansion due to the growth of conidiophores and conidia leads to the rupture of the cuticle or epidermis of the host, and the spores, which are usually of the slimy type, exude in masses and are splashed away by rain drops. The various genera of this group are mainly distinguished by the shape, color and septation of the spores, e.g., *Gloeosporium* with cylindrical, one-celled conidia (*G. fructigenum* causing bitter pit of apples, *G. musarum* causing spotting of bananas), *Marssonina* with two-celled conidia (*M. panattoniana* causing ring spot of lettuce), *Pestalozzia* with dark, septate spores, bearing characteristic appendages (Fig. 4l, m). The conidia of the Sphaeropsidales are formed on short conidiophores lining the inside of definite fruit bodies or pycnidia (Fig. 4k). These may be globose, as in *Phoma* and *Phyllosticta*, or flask shaped with definite necks opening by an ostiole, as in *Sphaeronema*. The majority of them form just below the surface of the host and break through when mature. Some are formed in or on stromata (e.g., *Cytospora*). Pycnidial and spore characters are used to distinguish the genera. As in the Melanconiales, the spores are usually slime spores and in moist weather they emerge through the ruptured wall of the pycnidium or the ostiole, when present, and often form a long spore "tendril" which is eventually splashed away by rain.

Among the conidial fungi we thus see many different methods of spore formation, all of which achieve the same end, namely the formation of large numbers of small dispersal units by which the fungus is able to spread. Despite the inevitable wastage of a large proportion of these spores, their small size and rapid production make them an economical as well as an efficient means of multiplication and spread of the fungus. The rapidity of formation and ease of dispersal of conidia is of particular value to plant pathogenic fungi, enabling them to take advantage of short periods of favorable weather conditions for spore production, dispersal, and germination of the spore, and consequent establishment of the fungus on or in a new host.

c. *Uredospores of the Rusts.* The uredospores of the rusts are functionally similar to conidia. These are formed in great numbers in small sori which break through the epidermis of the host. Uredospores are abstricted from the apex of a uredospore mother cell and in most rusts are shed immediately, but in the Coleosporiaceae they may occur in long chains. They are light, dry, wind-borne spores which are formed throughout the growing period in successive generations. Most rusts have yellow or reddish uredospores and the spore walls may be fairly thick. The uredospores of the cereal rusts, and perhaps others, are able to withstand the intense ultraviolet light of the upper atmosphere for long periods but do not survive the northern winter. They differ fundamentally from true conidia in being dikaryotic. They are produced only on the dikaryotic mycelium, are themselves binucleate, and give rise to further dikaryotic hyphae.

C. *Sexual Reproduction*

While the asexual spores of most fungi are mainly units of multiplication and dispersal, many, but not all, sexually produced spores have a different function, namely that of survival during a period of conditions unfavorable to vegetative growth. Sexual spores are resting spores or sedentary spores (Gregory, 1952). They are found in most lower fungi and in a few groups of higher fungi, such as the rusts and smuts. The basidiospores of the higher Basidiomycetes and the ascospores of many Ascomycetes are themselves propagules, but are commonly protected inside a more or less thick walled fruit body until maturity and are seldom shed except under conditions favoring both dispersal and germination.

1. *Sexually Produced Sedentary Spores of the Phycomycetes*

In the lower fungi, sexual fusion normally takes place at some stage in the life cycle, often as a result of the onset of conditions less favorable to mycelial growth and asexual spore formation. Fusion of sexual organs or gametes is usually followed immediately by fusion of nuclei from these and the development of a characteristic spore or spores. These often contain glycogen and oily materials as reserve foods and have a relatively thick outer wall impermeable to water and gases. They are usually incapable of germination until they are fully mature and until the wall is rendered permeable to water and gases, e.g., the oospores of *Phytophthora cactorum* (Blackwell, 1943). Alternate wetting and drying, or freezing and thawing, may crack the wall and end the dormancy of a resting or sedentary spore. Mechanical pressure, treatment with toxic substances, or passage through an animal's gut may have a similar effect, or the dormancy may end with increasing age of the spore.

Among the more primitive aquatic members of the Phycomycetes, such as the chytrids, sexual reproduction occurs by fusion of motile isogametes. Such an isogamous fusion occurs in the life history of *Synchytrium endobioticum*, the cause of black wart of potato. After fusion of two such free swimming gametes, the resulting zygote may come to rest on the surface of a potato tuber. It then withdraws its cilia and penetrates into the tissue, where it forms a single thick walled spore. This spore is set free into the soil by the decay of the infected tuber and it may remain there, dormant but viable for as long as 10 years before germinating to form a zoosporangium. Dormancy is thought to be prolonged by the presence of excess carbon dioxide in the soil. Many of the chytrids parasitic on green algae form resting spores in a similar manner, e.g., many of the aquatic Synchytriaceae (Canter, 1949), but others show a tendency to heterogamy, as shown by *Dangeardia mammillata* (Canter, 1946), where after two apparently similar gametes have come to rest on the surface of the host (the colonial alga *Eudorina elegans*) the contents of one pass into the other and a resting spore is formed.

The fusion of isogametes of *Plasmodiophora brassicae* (causing club-root of crucifers) in the root hair of the host is followed by the production of a small plasmodium. Resting spores are not formed until a later stage in the life history. Their formation is thought to be preceded by reduction divisions of the nuclei of the large plasmodia in the cortical cells of the host root.

In the saprophytic group of the Blastocladiales, sexual reproduction varies from the fusion of equal sized gametes to that of unequal sized ones of which the male contains orange carotenoid pigments.

The small aquatic group of the Monoblepharidales, containing the single genus *Monoblepharis,* is unique among fungi, since a passive female cell, or oosphere, contained in a more or less globose oogonium is fertilized by motile sperms released from an antheridium, the shape of which varies with the species. A zygote or oospore is then formed

In the large and mainly pathogenic group of the Oomycetes, a club-shaped antheridium applies itself to the spherical oogonium (Fig. 5a, b) and inserts a fine fertilization tube through which a nucleus passes to fuse with that of a female cell or oosphere. The fertilized oosphere secretes a wall, which in the more advanced genera is thick walled and highly resistant to drought and cold. In most species of the aquatic and mainly saprophytic Saprolegniales, or water molds, which are usually considered to be the most primitive group of the Oomycetes, each oogonium contains more than one oosphere and attracts to it a similar number of antheridia. The resulting oospores are relatively thinner walled than those of the higher Oomycetes, the pathogenic Peronosporales, where each oogonium contains only one oosphere. In the young oogonium of these more advanced forms, the cytoplasm is clearly differentiated into a central ooplasm, from which the oosphere develops, and a peripheral layer, the periplasm, which is used up during the maturation of the fertilized oosphere or oospore. Both ooplasm and periplasm are originally multinucleate but in most species all the nuclei of the ooplasm abort, except one which fuses with one of the antheridial nuclei. Blackwell (1943) showed that the young oospore of *Phytophthora cactorum* is unable to germinate until after an internal "ripening" process is completed and that germinating capacity is then limited by the low permeability of the thick wall. Sexual reproduction of the obligate parasites of the downy mildews takes place within the intercellular species of the host plants (Fig. 5c) and the oospore is set free only on the death and decay of the host. Oospores germinate by producing zoospores in the more primitive members and by a germ tube in the advanced ones.

Raper (1952, 1954, 1957) demonstrated conclusively that the various stages in sexual reproduction of the water molds, *Achlya bisexualis* and

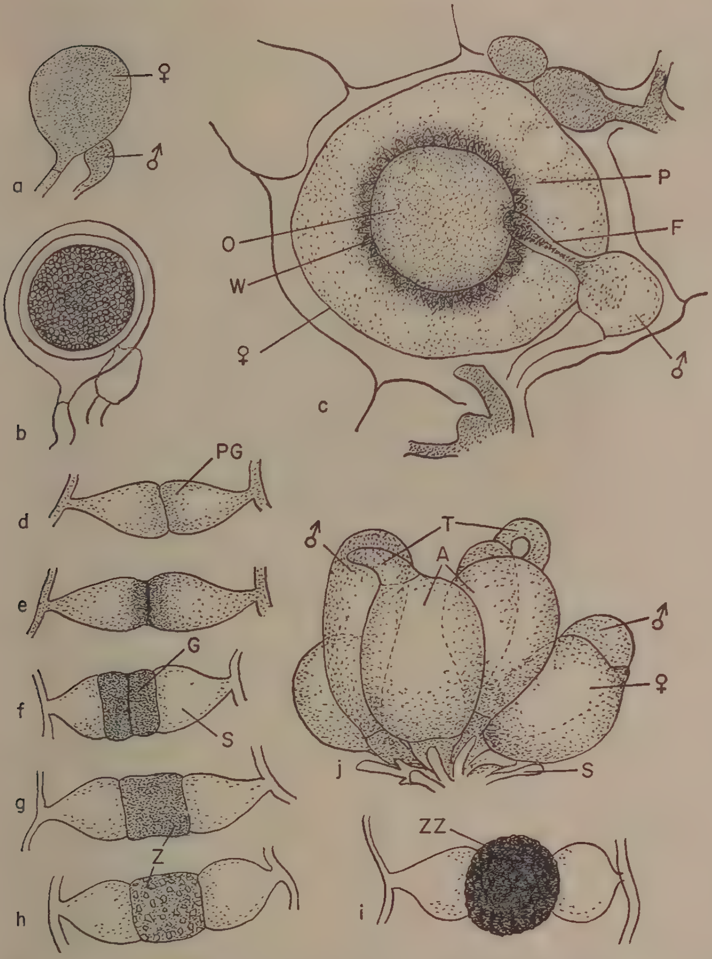

FIG. 5. Sexual organs of fungi: (a–b) *Phytophthora cactorum* (a) Young oogonium (♀) (contents not yet differentiated) and adjacent antheridium (♂). (b) Fertilized, thick walled oospore, empty antheridium, and remains of fertilization canal. (c) *Albugo tragopogonis*, oogonium (♀) and antheridium (♂) in intercellular space of host (*Tragopogon pratense*). F = fertilization canal, O = oosphere, W = developing wall, P = periplasm. (d–i) *Rhizopus sexualis* stages in conjugation and zygospore formation, PG = progametangia, G = gametangia, S = suspensors, Z = young zygospore, ZZ = mature zygospore. (j) *Pyronema confluens*, group of archicarps (♀) and antheridia (♂), T = trichogyne, A = ascogonium, S = sterile hyphae. Magnifications, a, b, c, j, × 550; d–i, × 80.

A. ambisexualis, are controlled by a series of unidentified specific hormones produced in turn by the female and male thalli. No such evidence has been obtained for hormonal control of sexual reproduction in the pathogenic forms but it probably exists in all of them.

In most of the Oomycetes, sexual reproduction takes place only after a considerable period of solely asexual spore production and probably as a result of a deterioration in the environment, such as a fall in food supply, the onset of low temperature, or of drought. The thick wall and dense cytoplasm of the oospore allows it to resist adverse factors which would be fatal both to the vegetative mycelium and to the delicate zoospores or even to the rather less vulnerable conidia of *Bremia* or *Peronospora*. Oospores are not dispersed actively but are set free in the place where they were formed, by the decay of host tissue or of the parent mycelium, and eventually germinate *in situ*. The oospore is thus a typical resting or sedentary spore whose main function is survival.

The remaining group of the Phycomycetes, the Zygomycetes, also produces a characteristic resting spore, the zygospore. In *Mucor*, *Rhizopus*, and a number of other genera, the zygospore is formed by the conjugation of similar sexual branches or gametangia. The two conjugating branches or progametangia approach one another, swell up, and finally the tips come into contact (Fig. 5d). The tips of these progametangia, the gametangia, then are cut off by cross walls from the supporting cells or suspensors (Fig. 5e, f). The walls between the gametangia break down and the contents of the two conjugating cells fuse (Fig. 5g, h). It is thought that all but one nucleus from each gametangium abort and that the remaining two fuse. The fused gametangia round off and become surrounded by a thick, darkly pigmented, and often warty wall to form the zygospore (Fig. 5i). In some other genera of this family the gametangia are of unequal size, or the contents of one may pass into the other. In *Mortierella* and *Endogone* the zygospores become surrounded by sterile hyphae to form small fruit bodies containing one or many zygospores, respectively. Many of this family show heterothallism, that is zygospores are not found on colonies developing from single sporangiospores but are formed only when two colonies of complementary strain, termed "plus" and "minus," are present. Hyphae from these colonies approach one another and conjugate in pairs of opposite strain. Homothallic species, in which conjugation takes place between hyphae of the same strain, are also common. The phenomenon of heterothallism was first discovered in this group by Blakeslee (1904) but has since been found to occur in most groups of fungi.

2. *Sexual Reproduction in the Higher Fungi*

The higher fungi seldom show such a clearly defined sexual phase as do the lower fungi. Even where definite sex organs are formed, except in a few relatively primitive groups of Ascomycetes, nuclear fusion and spore formation do not follow immediately after the fusion of sexual branches but are delayed until after a period of secondary vegetative growth, which may be particularly prolonged in the Basidiomycetes. The majority of species of both Ascomycetes and Basidiomycetes, however, produce no vestige of sex organs and the association of nuclei which precedes nuclear fusion is brought about by other means.

a. *Sex Organs of Ascomycetes.* In the simplest group of the Ascomycetes, the Endomycetales, sexual reproduction is prevalent and is typically of a simple type somewhat similar to that of certain Zygomycetes. Conjugation between adjacent cells of a hypha, as in *Eremascus* spp. and some species of *Endomyces*, or between two sprout cells of certain yeasts, such as *Schizosaccharomyces octosporus*, leads directly to the formation of an eight-spored or four-spored ascus. In some species of *Endomyces* the conjugating processes are of unequal size, thus showing a form of heterogamy, while in other species of this and related genera there is a strong tendency to the parthogenetic formation of asci. Among the yeasts there is a further tendency for the formation of asci to be delayed so that a secondary binucleate sprout mycelium, often consisting of "giant" cells, intervenes between fusion of cells and production of asci and ascospores. This reaches its limit in *Saccharomycodes ludwigii*, the ascospores of which conjugate in pairs while still within the ascus.

In the higher Ascomycetes, or Euascomycetes, which make up the bulk of the group, the association of nuclei, brought about either by the fusion of sex organs or by other means, is followed by a period of secondary vegetative growth. The ascogenous hyphae thus formed may show considerable branching and ultimately produce large numbers of asci. They are usually rapidly enveloped by sterile hyphae which form a protective layer around the developing asci. The resulting fruit body or ascocarp may be merely a weft of hyphae surrounding a group of asci, as in *Gymnoascus*, or may be a complex fruit body, the spherical cleistocarp of the Eurotiales and Erysiphales, the flask-shaped perithecium of the Pyrenomycetes or the variously shaped cups and even more complex forms of the Discomycetes. All these major groups include some members in which definite sex organs are formed. The male organ or antheridium is a single ovoid, club shaped, or more elongated cell borne on one or more stalk cells. The female branch, or archicarp, is usually more complex and in its typical form consists of a female cell or ascogonium borne on one or more stalk cells and bearing a receptive cell(s), the trichogyne, at the apex. The whole archicarp may consist of a row of as many as ten or twelve cells, but is usually much simpler. The trichogyne, when present, applies itself to the antheridium, as in *Pyronema confluens* (Fig. 5j) and the wall between them breaks down. The contents of the antheridium pass into the trichogyne and finally enter the ascogonium via a pore in the dividing wall. In the absence of a trichogyne, direct contact takes place between the antheridium and the ascogonium and the contents of the former pass into the latter (as in certain powdery mildews (Fig. 6r). True sexual fertilization of this type takes place in only a minority of species. In some species, as in certain strains of *Eurotium herbariorum*, the antheridium may form but aborts before fusion takes place. In a number of species from several widely separated groups, no antheridium is produced (e.g., *Ascobolus stercorarius, Humaria granulata*) although the ascogonium is apparently normal. Some such species possess a trichogyne and this may fuse with an asexual spore or oidium. In *A. stercorarius*, Bistis (1956, 1957) has shown that the trichogyne is attracted toward an oidium of suitable mating type by a chemical stimulus. The archicarp of *Humaria granulata* does not produce a trichogyne and the ascogonium is terminal. In the majority of species of the Euascomycetes neither antheridium nor archicarp is formed.

In a number of species, the microconidia function as male cells. The work of Drayton (1932, 1934) showed that apothecia were produced by *Sclerotinia gladioli*, the cause of dry rot of gladiolus corms (then known as *Sclerotium gladioli* and thought to be a member of the Mycelia-Sterilia) if microconidia of one strain were placed on sclerotium-like receptive bodies of another. Apothecia of *Sclerotinia* type were obtained by similar techniques for *Botrytis convoluta* (Drayton, 1937), *B. polyblastis* (Gregory, 1938a), *B. cinerea* (Groves and Drayton, 1939), *B. narcissicola*, and *B. sphaerosperma* (Gregory, 1938a, 1941). Genetic studies proved that with *Sclerotinia gladioli* the process of "spermatization" by the addition of microconidia leads to a true fertilization (Drayton, 1934).

Some members of both the Pyrenomycetes (e.g., *Gnomonia erythrostroma*, causing cherry leaf scorch; *Polystigma rubra*, causing a leaf spot of wild plum) and the Discomycetes (e.g., *Rhytisma acerinum*, causing tar spot of sycamore; *Lophodermium pinastri*, causing leaf fall of pine) produce pycnidia containing small spores which germinate feebly or not at all. It has been suggested that these spores, like the pycnidiospores of the rusts, may function as male cells or may once have done so. Whether such spores are primarily male cells or whether they acquired the function of male cells after the loss of the antheridium is a matter of speculation. Attempts to demonstrate the presence of a trichogyne in the fruit body initials of these species have not been wholly convincing. The presence of an ascogonium bearing a trichogyne and of pycnidiospores in certain ascolichens (e.g., *Collema* spp.) suggests that the species under consideration may once have had a similar type of fertilization.

Whether sex organs are produced or not, the formation of the ascogenous hyphae is preceded by the association of two nuclei of compatible type. In the absence of sex organs or of "spermatization" by microconidia, these nuclei are derived from vegetative cells. Buller (1941) and many later investigators have shown that the ascogenous hyphae are dikaryotic, that is they consist of binucleate cells. The two nuclei in a cell form a dikaryon and divide simultaneously, one daughter nucleus of each passing into each daughter cell at cell division. Thus the young asci are themselves binucleate and it is highly probable (and confirmed for an increasing number of species by genetic evidence) that these two nuclei are derived from the original paired nuclei, whether these were distinct male and female nuclei or pairs of uncertain vegetative origin.

In the unique group of the Taphrinales, which includes *Taphrina deformans*, the cause of peach leaf curl, and other plant pathogens, nuclei usually become paired by the conjugation of the ascospores or of yeast-like cells produced by the budding of these. The resulting dikaryotic mycelium eventually produces a number of binucleate ascus mother cells, the so-called chlamydospores, from which the asci develop.

b. *Formation of Ascospores.* Despite their varied form, globose, clavate, cylindrical, or even filiform (Fig. 6, k–g), the asci show a remarkable uniformity in development throughout the many and varied groups of the Ascomycetes. All asci begin as binucleate cells. These two nuclei fuse to form a large fusion nucleus as the ascus enlarges. This divides almost at once by a reduction division which, in most species, is followed by two more divisions to give eight nuclei. In some species, as in some of the hypogeous Tuberales, some of these nuclei abort giving an irregular final number. In a few other species, including some species of yeasts, only one or two divisions occur in the ascus. Whatever the final number of nuclei may be, the cytoplasm of the ascus becomes aggregated around these nuclei to form walled ascospores. This process of free cell formation is characteristic of ascospore formation. In *Taphrina*, the nuclei are situated only in the peripheral layer of the ascus.

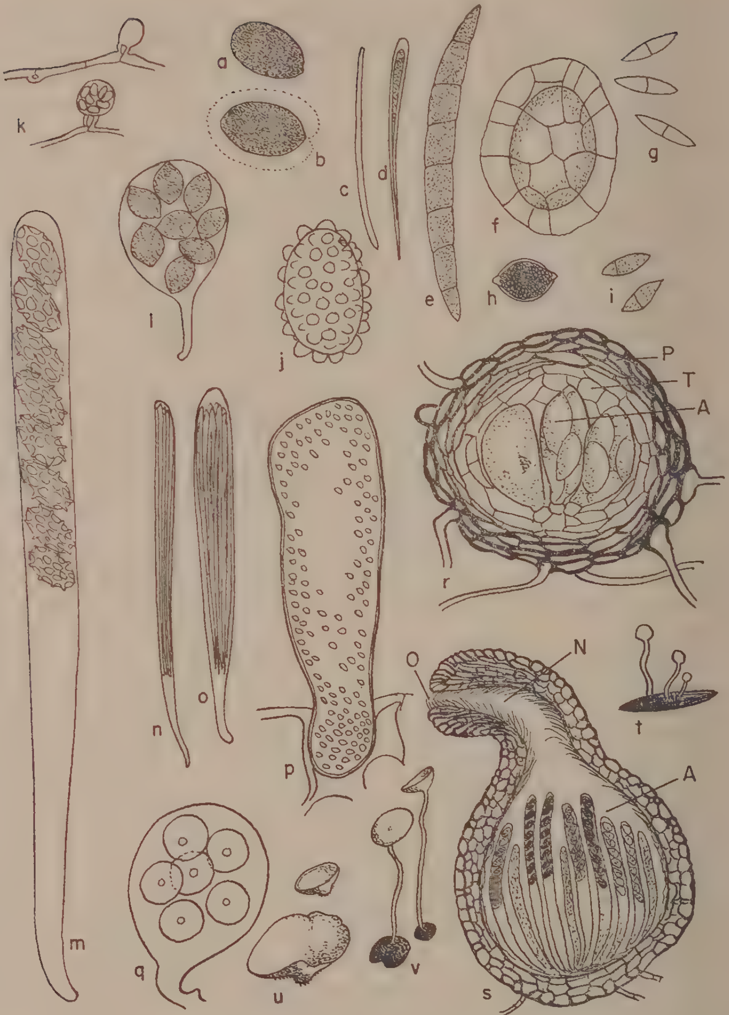

FIG. 6. Asci, ascospores and ascocarps: (a–j) Ascospores. (a, b) **Sordaria** *fimicola,* (b) showing gelatinous sheath. (c) filamentous spore of *Claviceps purpureum.* (d) *Rhytisma acerinum.* (e) *Geoglossum* sp. (f) *Tuber aestivum,* large spore with reticulate sculpturing. (g) *Nectria galligena.* (h) *Melanospora zamiae.* (i) *Stigmatea robertianum.* (j) *Genea hispidula,* hyaline warty spore. (k–r) Asci. (k) *Eremascus fertilis,* stages in formation of asci. (l) *Melanospora zamiae* (immature). (m) *Peziza aurantia,* cylindrical ascus with eight uniseriate, reticulately sculptured spores. (n) *Claviceps purpureum,* narrow ascus containing bundle of eight

The ascospores may be irregularly arranged in the ascus as in the Hemiascomycetes, Eurotiales, and Erysiphales and many Pyrenomycetes, or may be arranged in a single line in a cylindrical ascus, as in some Pyrenomycetes and most Discomycetes or, as in the filiform asci of *Claviceps purpurea* and other members of the subgroup Clavicipitales of the Pyrenomycetes, may be long and needle shaped and arranged in a bundle parallel to one another. They are most often one-celled and hyaline, but may be septate and even many-celled, pigmented, and with variously sculptured walls, or bearing gelatinous or filamentous appendages (Fig. 6, a–j).

Many plant pathogens reinfect their hosts in the spring solely by the production of clouds of ascospores, e.g., *Rhytisma acerinum*, causing tar spot of sycamore, and *Lophodermium pinastri*, which causes premature leaf fall of pine. The ascospores are usually formed in protective fruit bodies and thus are frequently not themselves resistant bodies. In some species, however, the ascospores are more or less resistant to adverse external conditions and may then act both as resting spores and dispersal units, as with some species of coprophilous Ascomycetes.

c. *The Formation of Fruit Bodies among Ascomycetes.* The asci of the Hemiascomycetes are formed singly, as in Endomycetales, or in a sheet or hymenium over the surface of the host plant, as in Taphrinales, and are not protected by any special aggregation of sterile hyphae. Those of the Euascomycetes are formed in groups or in hymenia and are more or less protected by sterile hyphae, the whole complex of sterile hyphae, ascogenous hyphae, and asci being termed a fruit body, or more precisely, an ascocarp. There is a wide variety of shape and complexity to be seen in the fruit bodies of the Ascomycetes, but the initial stages of development are surprisingly similar.

As already described (p. 143), ascogenous hyphae grow out rapidly from the fertilized ascogonium or from a binucleate mycelial cell and eventually produce asci. The ascogenous mass may be limited to a single short hypha producing only a single ascus, as in *Sphaerotheca*, and *Podosphaera* among the powdery mildews, or may develop into a

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filiform spores. (o) *Rhytisma acerinum*, also with spores arranged in a bundle. (p) *Taphrina deformans*, containing numerous spores derived from ascospores by budding (q) *Elaphomyces granulatus*, six-spored ascus. (r–v) Fruit bodies or ascocarps. (r) L.S. spherical cleistocarp of *Erysiphe polygoni*, showing peridium (P) of thick walled cells, inner tapetal layer (T), and asci (A) with developing ascospores, (s) L.S. flask-shaped perithecium of *Sordaria fimicola*, showing neck or beak (N) bent toward light, ostiole (O), and parallel asci (A). (t) Fertile stromata of *Claviceps purpureum* developing from sclerotium. (u) Cup-shaped apothecia of *Peziza aurantia*. (v) *Sclerotinia sclerotiorum*, sclerotia bearing stalked apothecia. Magnifications: a–q, × 600; r, × 400; s, × 120; t–v, × ½.

large much branched complex of hyphae producing numerous asci as in the Pezizales and Tuberales. In the more advanced species, asci are produced by a curious "crozier formation" from the penultimate cells of the ascogenous hyphae (Fig. 6r). In others, croziers are not seen.

At the same time as the ascogenous hyphae begin to develop, sterile hyphae grow up from the parent mycelium and envelop the ascogenous complex. These sterile hyphae may be limited to a thin weft surrounding the ascogenous hyphae and asci, as in *Gymnoascus* and other simpler members of the Eurotiales; they may be differentiated into a nutritive layer, or tapetum, which is used up in the development of the asci, and a definite closely interwoven outer wall or peridium, as in *Eurotium* itself; or the peridium may consist of several layers, as in the higher Pyrenomycetes and some Discomycetes (Fig 6, s–v). The cells of the peridial layer often become rounded, thick walled, and dark colored, and are usually pressed together so tightly that it is impossible to discern their hyphal origin.

Fruit bodies of the Ascomycetes show a wide range of form and structure, as shown in Fig. 6, r–v). Those of similar form may not always have developed in a similar manner [as with the Pyrenomycetes where modern research is showing that forms hitherto thought to be related differ fundamentally in the development of their fruit bodies (Luttrell, 1951; Munk, 1954, 1957)]. The simplest ascocarps are the spherical cleistocarps of the Eurotiales and Erysiphales. In those of the former group, the globose asci are arranged irregularly and break down early, releasing the ascospores into the cavity of the cleistocarp from which they are eventually set free by the decay or fracturing of the peridium. The cleistocarps of the Erysiphales show an advance in having, in most species, a more highly developed peridium, often bearing characteristic appendages, in the arrangement of the asci (in species having more than one) in a parallel layer or hymenium and, in many species (e.g., *Podosphaera leucotricha,* the apple mildew), in the possession of a definite spore discharge mechanism. The Pyrenomycete fruit body or perithecium shows a wide variation of the basic flask-shaped type. The neck may be vestigeal or absent, as in *Chaetomium,* short as in *Nectria* or *Sordaria,* or long and slender as in *Ceratostomella, Ophiostoma,* or *Venturia.* The perithecia may be scattered over the surface of the mycelium (e.g., *Sordaria, Neurospora*), embedded in host tissue (e.g., *Polystigma, Venturia*), on or partially embedded in a stroma (e.g., *Nectria*), which may itself be embedded in host tissue (e.g., *Phyllachora*), or they may be completely embedded in a stroma (e.g., *Claviceps, Xylaria*). In the Myriangiales and Pseudophaeriales, many of which were previously classified with the Pyrenomycetes, the asci are formed singly or in groups within locules or cavities in a stroma. Among Discomycetes, the extensive hymenium may be enclosed within the host tissue opening only to release the ascospores at maturity, (e.g., members of the Phacidiales such as *Rhytisma acerinum* and *Lophodermium pinastri*), or, more commonly, it extends over the surface of variously shaped fleshy or leathery fruit bodies. These may be the typical sessile cup-shaped apothecium (e.g., *Peziza*), a similar stalked structure (e.g., *Sclerotinia*), a club-shaped fruit body (e.g., *Geoglossum*), a stalked fruit body with a variously shaped fertile head (e.g., *Helvella, Morchella*), or a much infolded and complex subterranean structure (e.g., the truffle, *Tuber*).

Very few studies have been made of the pattern of growth in these complex ascocarps. The perithecia of the Pyrenomycetes are particularly difficult to observe owing to the rapid envelopment of the soft central parts or centrum by the tough or hard and brittle peridium. Progress has been made recently, toward an understanding of the various ways by which the centrum develops and the asci become arranged in it (Munk, 1954). Corner (1929a, b) studied the development of the comparatively simple cup-shaped apothecia of species of Pezizales and showed that marginal groups or fascicles of hyphae between the hymenium and the ground tissue or cortex of the fruit body grow out to extend the margin, so that the "cup" of the apothecium expands by marginal growth. In stipitate species, the stalk grows upward and its final length is determined by a phase of cell enlargement without further cell division.

The protection of the ascospores by the peridium of the fruit body has an obvious biological advantage. This, however, is accompanied by the disadvantage that dispersal of the spores is made more difficult. In the Eurotiales, ascospores are set free only by the decay or fracture of the peridium, but in the higher Ascomycetes, as will be shown in Volume III, Chapter 5, many complex mechanisms of dehiscence of the fruit body and discharge of the ascospores have been evolved.

d. *Sexuality in the Basidiomycetes.* We have seen (p. 143) that only a comparatively few species of the Ascomycetes produce functional sex organs, that in others one or both may no longer function, and that in the vast majority of species no sexual organs are formed at all. In the Basidiomycetes no trace of sex organs is to be found in any group except the Uredinales. In all other groups the association of pairs of nuclei of opposite mating type occurs as a result of the anastomosis of vegetative cells. Fusion of these nuclei is delayed until the formation of the basidia.

It has been known for a long time that the life cycle of the rusts includes both a uninucleate and a binucleate phase. The work of Blackman (1904) and Christman (1905) demonstrated that the binucleate condition originates by the migration of nuclei into the aecidiospore mother cells at the base of the aecidial initials. The aecidiospores are thus binucleate and this dikaryotic phase continues until nuclear fusion takes place in the young teleutospore. Blackman and others pointed out the resemblance of the pycnidiospores to spermatia in their small size, relatively large nucleus, poor germinating ability, their formation at the same time as the aecidial initials, and the production of sticky "nectar" by the pycnidia. A search for traces of a female receptive organ or trichogyne in the aecidial initials was unsuccessful. It was not until the brilliant and now classic work of Craigie (1931) that the function of the pycnidiospores was elucidated. Craigie and his co-workers have shown conclusively that most rusts are heterothallic and that the diploidization takes place by a number of methods. Where two mycelia of compatible strains of the same species meet and intermingle within the tissues of the host, the hyphae of these readily anastomose and interchange of nuclei follows. Diploidization can also be brought about by the fusion of a pycnidiospore, or spermatium, with a hypha of opposite strain projecting above the surface of the host. Migration and multiplication of the spermatial nucleus eventually leads to the diploidization of the aecidiospore mother cells, which was demonstrated by Blackman in 1904. Opinions differ as to whether the pycnidiospores are genuine spermatia or whether they are conidia which have assumed the function of the male cells. One might expect that if they had originated as male cells some trace of a corresponding female organ, probably possessing a trichogyne, would be found. They are, however, very similar to the pycnidia of certain lichens, e.g., *Collema* in which such female organs are still found.

No other group of Basidiomycetes has any trace of sex organs. In the Ustilaginales or smuts, a group which in many ways resembles the Uredinales, diploidization takes place either by the conjugation of the sporidia (basidiospores) or of yeast-like "conidia" developing from these, or by the anastomosis of vegetative cells at some other stage in the life history.

A number of species of the Eubasidiomycetes (Hymenomycetes and Gasteromycetes) are known to be heterothallic and a few have been shown to be homothallic. In the heterothallic species studied, the basidiospore on germination gives rise to a haploid, uninucleate, primary mycelium or this primary mycelium may at first consist of multinucleate cells. When such a monospore mycelium meets another of compatible mating type, anastomoses take place freely between the hyphae, followed by migration of nuclei leading to the development of a dikaryotic, secondary mycelium. This secondary mycelium may differ slightly from the primary one in vigor, mode, and amount of branching and in the presence of clamp connections. In some species, e.g., *Coprinus lagopus* (Brodie, 1931), it has been shown that a hypha of one strain may conjugate with an oidium of the complementary strain and a secondary mycelium may result. In homothallic species the dikaryotic condition arises autonomously by pairing of nuclei within the primary mycelium or, in some species, e.g., *Corticium tenestre* (Kneip, 1913), *Coniophora cerebella* (Kemper, 1937), and some Gasteromycetes, the dikaryotic state may arise in the basidiospore by division of the single haploid nucleus so that no primary mycelium is formed. Fruit bodies arise only on the secondary or dikaryotic mycelia. Fusion of the paired nuclei takes place in the young basidium and is followed immediately by a reduction division.

Thus the Basidiomycetes show a more prolonged dikaryotic phase than that in the higher Ascomycetes. The morphological difference between the haploid and dikaryotic hyphae of the Basidiomycetes is less marked than that between the haploid vegetative mycelium and the ascogenous hyphae in the Ascomycetes. The results of the prolonged dikaryotic phase in the Basidiomycetes are that (a) the whole fruit body is made up of dikaryotic hyphae, whereas that of the Ascomycetes is a composite structure in which the dikaryotic ascogenous hyphae are enclosed in a peridium composed of haploid hyphae and interspersed with haploid paraphyses; and (b) that a single nuclear pairing may give rise to a mycelium on which not one but many fruit bodies may be formed over a number of years, so that the number of spores resulting from a single cellular union may be enormous. It has also been suggested (Gäumann, 1952) that, by analogy with higher plants, the great morphological development and diversity of the higher Basidiomycetes may be the result of the increased importance of the dikaryotic phase in this group.

e. *Formation of Basidiospores.* The young basidium resembles the young ascus in being binucleate. The two nuclei fuse to give a single diploid nucleus which divides by a reduction division followed by a second division to give four haploid nuclei.

In the Eubasidiomycetes, the basidium is a single club-shaped or cylindrical cell. From the apex four, or less commonly two, pointed sterigmata grow up and the ends of these become inflated to form the basidiospore. The haploid nuclei wriggle through the sterigmata in succession, one nucleus passing into each spore. The basidiospores of the Hymenomycetes are arranged asymmetrically on the sterigmata (Fig. 7 c–d) and this, as will be described in Volume III, Chapter 5, is associated with the characteristic drop mechanism of spore discharge seen in this group. In the Gasteromycetes, spore discharge of this type does not take place and the spores are arranged symmetrically on the sterigmata. A few species of this group, e.g., *Tulostoma simulans,* have sterigmata arising laterally instead of apically on the basidium. Others, e.g., *Rhizopogon,* produce an indefinite number of spores on each basidium.

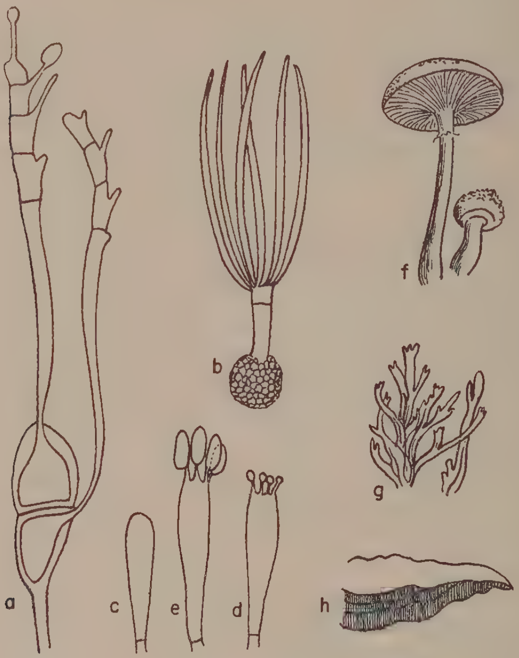

FIG. 7. Basidia and fruit bodies of Basidiomycetes: (a) *Puccinia graminis,* germinating teleutospore, producing promycelia (basidia) and sporidia (basidiospores). (b) *Tilletia caries,* germinating brandspore, producing short promycelium (basidium) and indefinite number of apical crescent-shaped sporidia (basidiospores). (c–d) Stages in development of holobasidium of *Coprinus sp.* Magnification: a–d, × 500. (f) Fruit bodies of *Armillaria mellea.* (g) fruit body of *Clavaria cinerea.* (h) T.S. fruit body of *Fomes annosus* showing layers of pores.

Most members of the Hymenomycetes have hyaline spores with smooth walls but others are pigmented and the walls, as in *Lactarius* and *Russula,* are variously sculptured. Buller (1922) has shown that the time necessary for maturation of pigmented or sculptured spores is much greater than that with colorless, smooth spores. Most species of Gasteromycetes produce colored spores which often show complex sculpturing of the wall.

The basidia and basidiospores of the rusts and smuts develop on germination of the teleutospores and smut spores, respectively. Nuclear fusion takes place in these spores before germination, and reduction division follows with the emergence of the basidium or promycelium from the germinating spore. The promycelium of the rusts is typically a short hypha of four cells, from each of which a sterigma develops and bears a sporidium or basidiospore (Fig. 7a) which is finally discharged by the drop mechanism as in the Hymenomycetes. In the smuts the situation is more variable. The promycelium may resemble that of the rusts and be septate and four-celled, as is usual in *Ustilago*, or it may be variably septate or aseptate, as in *Tilletia*. In *Ustilago*, sporidia may form in a manner similar to that already described for the rusts (but are not discharged by the drop mechanism) or the cells of the promycelium may give rise directly to a mycelium either with or without preliminary conjugation of the promycelial cells. In *Tilletia* and related genera, the sporidia are elongated and often sickle shaped and are formed in a group at the apex of the promycelium (Fig. 7b). Normally they fuse in pairs and give rise to conidia which are discharged by a drop mechanism similar to that of the basidiospore of the Hymenomycetes. The conidia of the smuts may undergo a yeast-like budding before the mycelium becomes established.

The small groups of the Auriculariales, Tremellales, and Dacryomycetales produce their basidia in a hymenium over the surface of variously shaped fruit bodies, as in the Eubasidiomycetes, but the basidia are transversely septate, longitudinally septate, and bifurcated, respectively.

The basidiospore, in all groups except some of the hypogeous Gasteromycetes, is a dispersal spore of a highly efficient type. Basidia and basidiospores are protected during development to a varying degree by the sterile parts of the fruit body in the higher Basidiomycetes. In the rusts and smuts germination of the teleutospores or smut spores usually takes place only under conditions favorable to the development of promycelia and sporidia so that the same end is achieved by different means and the basidiospores are launched under the most favorable conditions.

f. *The Formation of Fruit Bodies among Basidiomycetes.* The majority of species of the Eubasidiomycetes, together with the members of the Auriculariales, Tremellales, and Dacryomycetales produce their basidia in or on complex and often large fruit bodies (Fig. 7f–h). The range of fruit body form is much greater than in the Ascomycetes and some, such as the giant puff balls and the brackets of species of *Ganoderma*, reach a diameter of one or more feet and each produces millions of spores.

The form of the fruit bodies ranges from the simple cushion-like or irregularly lobed structures of *Dacryomyces* and *Tremella* or the flat or wrinkled encrustations of species of *Peniophora* and *Auricularia mesenterica* to the simple resupinate or bracket forms of *Stereum*, the more complex brackets of many polypores, the clubs of the Clavariaceae, the well-known pileate fruit bodies of the agarics, and the diverse and complex fruit bodies of the Gasteromycetes (falsetruffles, puff balls, earth stars, bird's nest fungi, stinkhorns, etc.). In many of these the area of the hymenium is increased by being spread over folds, spiny or flat laminate outgrowths, or the inside of tubular pores. Very large numbers of basidia and basidiospores are thus produced on a single fruit body, and as already pointed out, a single mycelium may produce numerous fruit bodies in successive crops over periods of many years; the age of large "fairy rings" has been calculated to be more than 100 years. The woody perennial fruit bodies of *Fomes* and *Ganoderma* produce a new layer of pores, within which the hymenium develops each year. It has been calculated (Buller, 1909) that a single fruit body of *Polyporus squamosus* may produce 11,000,000,000 spores, a giant puff ball 7,000,000,000,000, and even a large mushroom as many as 1,800,000,000 spores. When one considers the highly efficient spore dispersal mechanisms of these fungi (see Volume III, Chapter 5) their wide distribution is explained. Nevertheless, the wastage of spores is very great, since to maintain the population, less than one spore per fruit body would have to survive and form a new mycelium.

Most of these fruit bodies begin as small, more or less spherical, masses of undifferentiated interwoven hyphae. They become differentiated into their various parts at an early stage without any great increase in size. Enlargement of the fruit body then follows, largely as a result of the expansion of cells already formed, little further cell division taking place, as shown by Bonner *et al.* (1956) for the common mushroom. The final shape of the fruit body is achieved by differential expansion of the various parts. During this period of expansion of cells the dry weight of the fruit body continues to increase, showing that water and dissolved substances must be taken up from the substrate. By the method of hyphal analysis Corner (1932a, b, 1948, 1950, 1953) has shown that in the Clavariaceae and Polyporaceae the fruit bodies are made up of several different types of hyphae, each of which plays a part in the mechanics of the structure. The sterile hyphae of the large brackets of many species of the Polyporaceae are very varied and become strengthened by the deposition of a substance akin to lignin so that in many species of parasites of woody plants the fruit bodies are as hard and woody and almost as durable as the trunks of the host plants themselves.

g. *Biological Implications of the Loss of Sexuality among Higher Fungi.* Sexual reproduction among both plants and animals is recognized to be a highly efficient means of securing both increased variation and increased vigor. A high rate of variation is biologically of first importance among all organisms, and perhaps particularly so among pathogens where slight alterations in the structure or habits of the host may radically alter the environment of the pathogens. A recent study (Broyles, 1957) of races of *Puccinia graminis* has shown that many factors influence survival of this fungus and that no race combines all the characters known to have survival value. The most successful races are those possessing a number of such factors. Here hybridization, by which new races arise, is obviously a factor of great biological significance in securing the success of the pathogen.

The clearly defined sexual phase in the life cycle of most Phycomycetes probably provides the advantages of variation through rearrangement of nuclear material and, in addition, frequently offers a means of surviving temporarily adverse conditions through the production of resting spores. In many Ascomycetes, the sexual phase is equally clearly marked but, as described in the previous sections, these are in the minority and in most Ascomycetes and nearly all Basidiomycetes sexual organs are no longer formed. This loss of definite sexual organs might well have meant disaster had it not been for the facility with which cells of the same species anastomose and their nuclei migrate. While in many Ascomycetes the association of male and female nuclei in the ascogonium has been replaced by the association of nuclei from vegetative cells of the same mycelium, in many others and in many Basidiomycetes the phenomenon of heterothallism ensures that these pairing nuclei shall be derived from mycelia of opposite and compatible strains. Thus the advantages of a fusion of nuclei derived from different sources and the subsequent segregation of characters during reduction division, are retained even when sex organs are no longer produced.

While the modified form of pairing of nuclei, followed by fusion and reduction division, seen among the Ascomycetes and Basidiomycetes, has apparently all the advantages of a sexual phase, there are many higher fungi, the Fungi Imperfecti, in which neither ascospores nor basidiospores are produced and in which no trace of a true sexual nuclear fusion is to be seen. Nevertheless it has long been a commonplace that many members of the Fungi Imperfecti are highly variable. The studies of Brown and his co-workers with *Fusarium fructigenum* (Brown, 1925, 1926, 1928; Brown and Horne, 1926) showed the extent of variation in a single "imperfect" species and indicated that the phenomenon of "sectoring" in plate cultures (where a sector of the colony shows distinct differences from the parent strains) must be due to genetic changes at the point of origin of the sector or "saltant." Pontecorvo and Gemell (1944) further examined the implication of sectoring which is now known to be due to genetic changes at the point of origin. Hansen and Smith (1932) introduced the concept of heterokaryosis to explain the high degree of variation in *Botrytis cinerea* and other Fungi Imperfecti. They showed that anastomosis between mycelia of the same species takes place freely and is not limited to strains of particular mating type. By such anastomosis and subsequent migration of nuclei from one hypha to another the multinucleate vegetative cells of such a fungus as *Botrytis* contain nuclei of different origin. When the conidia are formed, the proportion of the various nuclei which they contain is a matter of chance. With a large spore, such as that of *Botrytis* which may contain fifteen to twenty nuclei, the range of possible variation in the nuclear content is considerable and is reflected in the great degree of variation between colonies grown from single conidia. In species, such as *Verticillium*, in which the conidia contain few nuclei, or only one, the degree of variation due to heterokaryosis of the parent mycelium is correspondingly lower. Pontecorvo *et al.* (1952) showed that a recombination of hereditary factors takes place in the saprophytic *Aspergillus nidulans* by a process which they termed "parasexuality." This involves the actual fusion of two haploid nuclei in a heterokaryotic cell and their subsequent multiplication side by side with the remaining haploid nuclei. A diploid strain may become established from such material and segregation of characters may occur by mitotic crossing over and the occasional vegetative production of haploid nuclei by the diploid strain. It has since been shown that the parasexuality occasionally occurs in other fungi, including the pathogenic *Fusarium oxysporum* (Buxton, 1956). Buxton showed conclusively that variation in virulence in this species resulted from heterokaryosis and parasexual recombination. The part played by such nonsexual mechanisms of rearrangement of nuclear material in determining the ability of a fungus to overcome host resistance is obviously of primary importance. Loss of true sexuality is thus clearly of little significance among fungi, since owing to the ease of anastomosis, nuclear migration, and chance fusion of nuclei of different origin, they may be said to be largely independent of sex.

D. *The Effect of Environment on Reproduction of Plant Pathogenic Fungi*

When a pathogen is established inside the host, it is probably not greatly influenced, by fluctuation of the environment external to the host, with the possible exception of changes in temperature, except indirectly through the effect on the chemical and physical state of the plant cell or intercellular spaces. After a preliminary vegetative phase, during which the host is exploited to a greater or lesser extent, most plant parasites enter into a reproductive phase. No doubt this change is largely due to changes taking place within the thallus of the parasite, as a result of the accumulation of nutrients absorbed from the host, but it is also likely that the condition of the parasitic hyphae is influenced by the condition of the host. Many parasitic fungi, representative Phycomycetes, Ascomycetes, and Basidiomycetes, produce their sexual stages in autumn at the end of the growing season of the host, when it is reasonable to suppose that the food supply in the host is depleted. It is well known that a fall in food supply often induces fruiting in fungi in culture and it is highly probable that the same effect is seen in the host plant in autumn. The seasonal fall in temperature, however, may also play a part. The fall in food supply is the more likely explanation of the prevalence of sexually produced spores in autumn. This is supported by the fact that many pathogenic fungi produce these spores when the available food supply has been depleted by their own activity, irrespective of changes in temperature.

The distribution of nutrients in the host probably determines the site at which sporulation will take place. The formation of smut spores only in ovules or young seeds and anthers, by the grain and anther smuts respectively, strongly suggests that some food substance, possibly of a vitamin nature, is present in ovules or anthers at a favorable concentration to induce spore formation.

A reduction in and a change in the nature of the food supply in fallen leaves is probably important in inducing the formation of fruit bodies, e.g., perithecia of *Venturia*, and apothecia of *Lophodermium* and *Rhytisma*, in these after they are shed. Similarly, fruit bodies of many wood-destroying polypores form only when the interior of the tree has been almost consumed.

The production of toxic or antibiotic substances by the host may check spore formation. The well-known darnel grass fungus shows so many resemblances to the smuts that it is often assumed to be an imperfect smut and it is possible that the failure to produce spores is a result of unsuitable conditions within the host. Similarly the orchid endophyte is maintained in a vegetative condition within the root and finally undergoes lysis. Burges (1938) showed that lysis could be brought about by the sap of the host cell. Gaumann and Jaag (1945) showed that tubers of certain orchids produce substances capable of checking the growth of the endophyte. A similar antibiotic effect of bulbs of *Allium ursinum* on the arbuscular-vesicular endophyte *Pythium ultimum* was demonstrated by Hawker *et al.* (1957). It is highly probable that such antibiotic substances check spore production even more readily than vegetative growth of the pathogen.

Most asexual spores either do not form within the host or mature only on exposure at the surface. The sporangiophores of *Phytophthora infestans* and of the other downy mildews grow out through the stomata and produce sporangia (or conidia) after emergence. The same is true of the conidiophores of many Hyphomycetes. The pycnidia of most members of the Sphaeropsidales, the acervuli of the Melanconiales, the aecidia and uredosori of the rusts develop within the host and break through to the surface when nearly mature. The internal state of the mycelium must play an essential part, but weather conditions are also important, at least during the final emergence of the spore-bearing hyphae. Some asexual spores, such as the sori of "summer sporangia" of *Synchytrium endobioticum*, *S. taraxaci*, and many chytrids parasitizing green algae, develop within the host at a particular physiological stage of the parasite, apparently independently of the external environment.

In the present state of our knowledge, we can only speculate about the factors within the host and within the pathogen itself which lead to the onset of reproductive phases of various types and the initiation of spores. With the emergence of the pathogen at the surface of the host, it should be possible to arrive at a more accurate picture of the factors influencing the development and maturation of spores and spore-bearing structures. The development of spore-bearing structures outside the host is obviously influenced by the temperature and humidity of the air, by charges in these, and by stillness or movement of the layer of air immediately above the host surface. Light also plays a part with some species. Unfortunately the accurate measurement of conditions in the "microclimate" immediately surrounding fungus and host surface is extremely difficult. It has been shown that this microclimate may differ considerably from the conditions obtaining in the outer air. Much relevant information is scattered throughout the literature of plant pathology but has not been collected together to form a coherent story. A few examples only will be given.

It is well known that the range of any particular factor permitting sporulation is usually less than that for mycelial growth. This is a useful character, since spores are formed only under conditions allowing a margin of safety for the establishment of a new mycelium after germination.

Air temperature is an important limiting factor in the development of spores outside the host. Within the range permitting production of spores by a particular species, temperature influences not only the number of spores or fruit bodies produced but the type and morphology. *Phytophthora infestans*, owing to its economic importance, has received much attention and the conditions leading to intense sporulation are so well known that a blight forecasting system, based on weather reports, is in operation in a number of countries. Night temperature in late July and August (under conditions existing in England) is of great importance. The sporangiophores develop freely only on warm nights with a high relative humidity. In England, the oak mildew, *Microsphaera quercina*, produces cleistocarps, only in unusually hot summers (Robertson and Macfarlane, 1946), few or no teleutospores are formed by *Puccinia antirrhini* in cold years but large numbers were formed in the abnormally hot summers of 1955 and 1959. The size of spores and spore-bearing structures may also be influenced by temperature; the sporangia of *Choanephora cucurbitarum* increase in size with increase in temperature (Barnett and Lilly, 1950); the conidia of *Peronospora parasitica* are of average size $23 \times 19.5\mu$ at 20° C. but as much as $27 \times 23\mu$ at 5° C. (Thung, 1926); both temperature and humidity influence the length of sporangiophores of *Pseudoperonospora humuli* (Arens, 1929).

Humidity of the air is also of particular importance, as in the formation of sporangiophores of *Phytophthora infestans* which has already been mentioned. Orth (1937) showed that development is checked by very brief exposure to as little as 5% below optimum humidity. Many other fungal plant pathogens are equally dependent upon a relatively high humidity during the critical period of the development of sporangia or conidia. The downy mildews sporulate only in humid air, the conidia and conidiophores of *Peronospora destructor* (onion mildew) develop over the range 90–100% relative humidity (Yarwood, 1943), those of *Bremia lactucae* (lettuce mildew) develop only over the narrower range 98–100% relative humidity (Ogilvie, 1944), and abnormally pale plants of *Blackstonia perfoliata* in the field normally show no other external signs of infection, but become covered with a thick felt of conidiophores of *Peronospora chlorae* when kept under a bell jar in the laboratory (Fraymouth, 1956). Many other groups, notably the Hyphomycetes, such as *Botrytis cinerea* and *Cladosporium fulvum*, which, like the Peronosporaceae, provide no protection for their conidiophores, are equally sensitive to air humidity. The powdery mildews, in contrast, sporulate best at a slightly lower relative humidity and spore production may even be checked at 100% saturation. *Sphaerotheca humuli* var. *fuliginea* produces abundant conidia at a relative humidity of 93–96%, but the number falls off at higher humidities (Hashioka, 1938). Small fluctuations in humidity have a much more marked effect than quite large fluctuations of temperature within the range permitting sporulation. While practically all species producing delicate and exposed spore-producing hyphae require a high atmospheric humidity for emergence from the host tissue and further development, some require a slight fall in humidity for the final maturation of the spores. Byrde (1953) demonstrated that such a slight fall is necessary for the rounding-off of the conidia of *Sclerotinia* (*Monilia*) *fructigena*. In contrast, Cheal and Dillon-Weston (1938) showed that an actual film of water is essential for the budding-off of the conidia of *Venturia* (*Fusicladium*) *pyrina*. It is obvious that beyond stressing the general requirement of a humid atmosphere, no generalization can be made and each species must be studied individually.

The effect of humidity on type and form of sporing structures is illustrated by the prevalence of sporangia of *Choanephora cucurbitarum* at a relative humidity of 100% at 25° C. and over, and of conidia at lower humidities (Barnett and Lilly, 1955), by the increased length and septation of conidia of *Ramularia vallisambrosae* in relatively dry conditions (Gregory, 1938b) and in the well-known reduction in length of sporangiophores and conidiophores of a variety of fungi when grown under conditions of reduced humidity.

Maximum cell multiplication of bacterial plant parasites and the consequent exudation of bacterium-containing liquid is also dependent on wet conditions.

The tendency of spores, fruit bodies, and sclerotia of many fungi to form in concentric rings is well known. Experiments by many independent investigators have shown that, in artificial culture, this is most often due to diurnal alternations of light and darkness. Perhaps the best known example of a plant pathogen showing the dependence of sporulation on light is *Sclerotinia* (*Monilia*) *fructigena*, but Yarwood (1936, 1937, 1941) has shown that the daily periodicity of sporulation and spore discharge in *Erysiphe polygoni*, certain downy mildews, and *Taphrina deformans* is also largely a result of alternating light and darkness. Other less obvious effects of light have been recorded, such as the formation of numerous perithecia of *Diaporthe phaseolorum* (Timmick *et al.*, 1951) in alternating light and dark, the formation of only a few perithecia containing numerous ascospores in continuous darkness, and of numerous perithecia containing few or no spores in continuous light. The relative numbers of macroconidia, microconidia, and chlamydospores, and the length and degree of septation of the macroconidia of species of *Fu-*

sarium are all influenced by light (Harter, 1939; Snyder and Hansen, 1941; Brown, 1926; Carlile, 1956).

The effect of light on sporulation of many species is stimulatory but in other, often closely related, species the reverse may be true, e.g., light stimulates conidial formation in *Sclerotinia fructigena* but reduces it in *S. fructiola* (Hall, 1933); formation of pycnidia of some leaf spotting fungi is induced by light and of others is repressed (Leonian, 1924). Again, as with humidity, no generalization can be made concerning the effect of light.

Other factors such as the accumulation of volatile fungicides in still air, the presence of other organisms, etc., also play a part in sporulation at the surface of the host. Sporulation at the surface of subterranean parts of plants is particularly influenced by the rest of the soil flora and fauna and also by the rate of accumulation and diffusion of gases such as carbon dioxide.

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CHAPTER 5

Spore Germination

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Except for the sexual function of certain specialized spores, the spore may be defined as a specialized microscopic structure capable of initiating new growth. Morphological differences are well known, as is the difference in origin between sexual and asexual spores. Functionally there is again some diversity: survival of the fungus over a period of time when growth is impossible and dispersal of the organism in space are the two major roles of spores. A more narrowly reproductive function is fertilization, diploidization, or other nuclear transfer; a spore may be limited to this function, as is the rust fungus pycnospore, or nuclear transfer may be only one of its potentialities, as exemplified by the microconidia of certain Ascomycetes.

By comparison with spores, sclerotia as organs of survival or reproduction and rhizomorphs as organs of local spread are relatively insignificant, however important they may be for particular species. To a certain degree, these organs bear a functional resemblance to spores; the sclerotia of *Claviceps purpurea*, for example, germinate only if exposed first to low temperature (Garay, 1957), and the rhizomorphs of root pathogens function, like spores, in the establishment of new infections (Garrett, 1956).

Most studies on spore germination employ as criterion the final germination of a population; other criteria have been used much less. The latent period of germination, i.e., the time required for germination to begin, responds somewhat differently to temperature (Cochrane, 1945a) and to some toxicants (Tomkins, 1932). Germ tube elongation may or may not correspond in its reaction to total germination; one would expect, *a priori*, that it would resemble mycelial growth rather than germination, but the evidence for a fundamental difference is not strong.

Naturally enough, much of our information on spore germination relates to the effects of environmental factors on economically important fungi. This kind of study combines the merits of practical importance and easy measurement. However, concentration on such studies has meant a relative neglect of fundamental physiological problems. For example, the dormant or ungerminated spore is probably differentiated metabolically from the germinating spore, and the differences might cast some light on the general biological properties of growing as distinguished from resting cells. Germinating spores, in principle at least, offer the best material for the study of several aspects of the metabolism of fungi; it is well known that a population of mycelial cells contains a large and usually undetermined number of senescent or dead cells and that the living cells of necessity range across the whole span of developmental status. The control of nuclear number in spores by environmental conditions (see below, Section I) affords us a tool for the study of the role of the nucleus in metabolism and development. The responses of spore germination to light, discussed later in this chapter, may, if studied thoroughly, make significant contributions to radiation biology; in particular, the stimulation by light of a heterotrophic organism promises to open up new areas of understanding.

Spore germination has been reviewed intensively by Gottlieb (1950), and in less detail by the present author (Cochrane, 1958). In this chapter the intention has been to supplement rather than replace these reviews and to organize the material more specifically toward the problems of plant pathology. Naturally, we cannot afford to neglect saprophytes, since it is probable that most of the phenomena of spore germination are common to both pathogenic and other fungi.

I. The Germination Process

It has become customary, and in the present state of our knowledge it is no doubt necessary, to consider all types of spore germination as fundamentally alike. One spore forms a germ tube, another releases motile swarmspores, a third puts out a specialized promycelium with basidiospores, and a fourth germinates by cutting off secondary spores. We will not challenge the heuristic value of lumping all of these phenomena together, but it is necessary to realize that some oversimplification is involved. The common observation that for spores with two alternative modes of germination the environment often determines which is to prevail indicates that there are physiological differences between the germination types and that they are not in fact alike. Basidiospores of *Rhizoctonia solani* form secondary spores on water agar, germ tubes on a nutrient agar (Hawn and Vanterpool, 1953). The asexual spores of *Phytophthora infestans* form a germ tube at high temperature, endogenous swarmspores at low (Crosier, 1934). It would be interesting indeed to determine the precise nature of the stimuli at the cellular level which determine and direct morphogenesis in these forms.

Conidia of the higher fungi may be uni-, bi-, or multinucleate at the time of their formation (Bellinger, 1956; Hansen, 1938). The nuclear situation is of greatest importance in relation to genetic variability—it is obvious that a single-spore culture will or will not be genetically uniform depending on whether the original spore contains nuclei of dissimilar origin. However, the observation that a spore is multinucleate does not necessarily mean that the nuclei are genetically different; conceivably, the nuclei can arise by simple mitosis from a single original nucleus present in a uninucleate conidiophore. Conversely, a heterokaryotic mycelium which produces uninucleate conidia essentially regenerates the parent types during sporulation, unless there has been gene exchange in the heterokaryon between the component nuclei.

The number of nuclei in macroconidia of *Neurospora crassa* is reduced by cultivation in a minimal medium, increased by provision of amino acids (Huebschman, 1952), and reduced by cultivation with sorbose (Atwood and Mukai, 1955). In general, it appears that the ratio of deoxyribonucleoprotein to cytoplasm is held constant in multinucleate spores by variations in the over-all size of the spore (Huebschman, 1952; Ishitani *et al.*, 1956).

Morphologically, three distinguishable events characterize the process of spore germination in the asexual spores of the higher fungi. First, there is nuclear division at some time during the process (Bellinger, 1956). Second, the spore is usually observed to swell considerably. This is general, but not universal in fungi; spores of *Uromyces fallens* do not swell, and observations on *Sclerotinia fructicola* conflict (Mandels and Darby, 1953; Yarwood, 1936b). As discussed later (Section VI), it has been suggested that swelling is simply uptake of the water necessary for growth and development. Finally, a germ tube appears.

The time course of germination has been very thoroughly studied in relation to the use of spore germination assays for fungicidal activity (Wellman and McCallan, 1942). A plot of the probit of per cent germination (corrected for nonviable spores) against the reciprocal of time is linear; that is, the time of appearance of the germ tube is normally distributed against time. Thus, the observed spread in time between appearance of the first and the last germ tube in a population of spores is an expression of random variability. The rate of germination and the time of appearance of the earliest germ tubes is a species character, varying from less than an hour to 24 hours or more (Bennett, 1921; Gottlieb, 1950).

Germ tube growth presumably follows the same course as mycelial growth; extension occurs only at the tip, and, in most fungi, growth is exponential for a considerable period (Pratt, 1936; Smith, 1924; Stadler, 1952). Tropisms toward water and toward nutrients have been reported —the evidence is reviewed by Brown (1936)—but the situation is still unclear. The negative tropisms toward light and toward other germ tubes are considered later in this chapter.

II. Maturation and Dormancy

Gottlieb (1950) defines a dormant spore as one which "does not germinate under the same nutritive and environmental influences which later allow production of germ tubes." This definition is perhaps the best which can be phrased in the present state of our knowledge, but, as its author realizes, it probably includes more than one fundamentally different process.

Urediospores of the rust fungi germinate less rapidly and less completely if they are removed from the sorus before they have separated from their parent cells (Zimmerman, 1925). Spores so separated will, however, become germinable in 24–48 hours (Cochrane, 1945a). Formally, these prematurely collected spores fit the definition of dormancy just given. Since, however, the period needed for acquisition of full germinability is so short, and since spores collected from a mature sorus germinate immediately, it seems appropriate to consider the phenomenon one of immaturity and maturation. Whether the prolonged period of poor germinability of aeciospores of *Gymnosporangium* spp. (MacLachlan, 1936) can be described in the same way is not certain.

The thick-walled resting spores (or sporangia) of the Phycomycetes often fit into the concept of dormancy in that an apparently mature spore is unable to germinate for some time. At least part of this period probably represents the time required for cytological processes, and again might better be described as maturation. There may be, after the completion of all cytological events, a period of physiological dormancy (Blackwell, 1943). This is indicated first of all by the extreme length of the obligatory rest period of some forms—up to years in *Peronospora destructor* (McKay, 1957). More crucial evidence for a physiological dormancy is that a number of treatments shorten the rest period: chilling is especially effective for several forms (Arens, 1929; Blackwell, 1943; Schlösser, 1929), but so, for one species, is a treatment with permanganate in the presence of organic matter (McKay, 1939). The rest period of resistant sporangia of *Allomyces* spp. is shortened by cultural conditions and by indoleacetic acid (Machlis and Ossia, 1953).

The situation in the Phycomycetes is thus not yet clear. Perhaps the most useful working hypothesis is that the obligatory rest period—which, of course, is not universal in the group—is in part occupied by cytological events and in part by a more physiological dormancy. Attention should be directed to the possibility that the thick cell wall itself contributes to dormancy and must be altered before germination becomes possible. This approach is suggested particularly by work in the Blastocladiales, indicating that cultural conditions which cause the resistant sporangium to have an abnormally thin wall concomitantly shorten or abolish the rest period (Machlis and Ossia, 1953).

The teliospores of the rust fungi and the chlamydospores of the smut fungi pose much the same problems. These too are often but unpredictably accelerated in their germination by chilling, by treatment with miscellaneous chemicals, or by soaking. Here again, a survey of the literature suggests that the most promising hypothesis is that the heavy cell wall contributes to dormancy.

The ecological advantage of the resting spore is obvious, at least to plant pathogens which must survive a cold winter. It is also apparent that some heterogeneity in the time of ending of dormancy would have survival value for a species dependent upon a particular host which may not always be present in the area. Finally, the stimulating effect of root excretions on the germination of dormant spores offers a third mechanism for survival, in that germination could in principle occur only in the presence of a host (Garrett, 1956).

Experimentally, perhaps the most promising approach is the treatment of dormant thick-walled spores with various enzymes—lysozyme, ficin, hemicellulase, cellulase, chitinase, etc.—which might change the structure or permeability of the outer cell wall. These enzyme preparations are now easily available.

Ascospore dormancy has been studied intensively in *Neurospora tetrasperma*. Dormancy is broken by brief heat treatment or by furfural and related compounds; only the heat activation is reversible (Emerson, 1948; Goddard, 1939; Sussman, 1953a, b). So far, the cause of the dormancy has not been determined; respiratory changes occur but cannot be said to be primary. Recently, evidence has been presented which tends to implicate permeability as at least a major difference between dormant and activated spores: certain respiratory poisons do not enter dormant spores in amounts sufficient to inhibit respiration after activation, but do enter activated spores (Sussman *et al.*, 1958). Some association of cell membranes with dormancy is indicated by the activating effect of alkali on ascospores of *Ascobolus* spp. (Yu, 1954), and by the finding of Brierley (1917) that ascospore dormancy in *Onygena equina* develops only after the heavy spore wall is laid down.

III. Spore Longevity

From any survey of the literature on the survival of fungi under natural and artificial conditions, the first impression is of the extreme differences between types of fungi. The conidia of the powdery mildew fungi, sporidia of rust fungi, and sporangia of downy mildew fungi live for periods of only days under the most favorable conditions. At the other extreme, the survival of chlamydospores of the Ustilaginales, resting spores of the Phycomycetes, and basidiospores of some Hymenomycetes may be measured in years. The survival of herbarium specimens for as long as 21 years (Zobl, 1943) is presumably to be attributed to spore longevity.

Laboratory studies may, however, give a misleading impression. Among the rust fungi, for example, survival of urediospores in nature is shorter than that under conditions chosen in the laboratory as favorable (Cochrane, 1945a; Rosen and Weetman, 1940).

As a rule, sexual spores survive longer than asexual; however, the conidia of some imperfect fungi may survive very long periods (Roberg, 1948). From an evolutionary point of view, species which have lost their sexual stage probably disappear unless their asexual spores—or other enduring organs such as sclerotia—can take over the function of carrying the organism over long periods of unfavorable conditions.

As will be seen, the literature on longevity is almost entirely descriptive; events at the cellular level can only be guessed. One finding might be mentioned, that conidia of nutritionally deficient mutants of *Ophiostoma multiannulatum* die less rapidly in a starvation medium than do spores of the parent stock; presumably, the deficient spores metabolize less and hence survive longer than the nondeficient (N. Fries, 1948). This clue points toward a possible generalization that external factors influence longevity, at least in part, by their effect on endogenous metabolism, on the rate of disappearance of reserve materials or of cofactors essential for germination. If this is correct, it follows that short-lived spores have a higher rate of endogenous metabolism, on a dry weight or protein basis, than more durable spores; this supposition has not as yet been tested.

A. *Humidity*

Humidity and temperature as factors in longevity can be separated for convenience only. The two environmental influences in fact interact in any given natural or experimental situation. Nevertheless, one factor or the other may exert so strong an influence that it can be said to be isolated; thus, whatever the temperature, conidia of *Monilinia fructicola* survive longest at 75% relative humidity (Naqvi and Good, 1957). A number of other fungi are also favored by an intermediate humidity; this includes, for example, the urediospores of several rust fungi (Cochrane, 1945a).

However, humidity relations are not the same for all fungi. A number of rather short-lived spores—sporidia of rust fungi, conidia of the powdery mildew fungi, and sporangia of *Phytophthora infestans*—survive longest at saturation and are less viable at any lower relative humidity (Hyre and Cox, 1953; Longrée, 1939; Spaulding and Rathbun-Gravatt, 1926). We may suppose that the short life of these spores is the consequence of their inability to stand desiccation.

Finally, the spores of a number of fungi survive longest at low relative humidity; the effect is of course more marked at high than at low temperature (Merek and Fergus, 1954; Naqvi and Good, 1957). Presumably, in these spores, the loss of water is not injurious and, once water is largely removed, the chemical processes of senescence become much slower.

The real puzzle is that type of spore which is favored by intermediate humidity. It is possible, of course, that two opposing processes simply balance at an intermediate point, but the explanation is not really satisfying—a unitary theory would be preferable but cannot at present be framed.

B. *Temperature*

One of the few generalizations we can make is that down to the freezing point spore longevity is an inverse function of temperature; survival is longest at or near 0° C. The effect of higher temperatures is presumably compound: at moderate temperatures and high humidity endogenous metabolism may be accelerated and result eventually in exhaustion of stored reserves. At still higher temperatures or under drying conditions, excessive dehydration or protein denaturation may be more important. However, it should be stressed that these are speculations only; the data available on this topic are almost exclusively descriptive.

The death of fungi at very high temperatures has been studied relatively little. The thermal death point—the least temperature at which a population of cells is killed in 10 minutes—is about like that of the vegetative cells of mesophilic bacteria, i.e., 40–60° C. (Ling and Yu, 1941). However, the thermal death point and the related thermal death time are not particularly useful, since other conditions—notably the water content of the spores and the relative humidity of the atmosphere—affect the value markedly and may vary from one determination to another (Cole and Fergus, 1956; Groom and Panisset, 1933; Jensen, 1948).

The order of death at high temperature is usually not exponential (Hull, 1939; Smith, 1923; Williams *et al.*, 1941). The sigmoidal curve obtained when the fraction surviving is plotted against time of heating probably is to be interpreted as reflecting variability in heat resistance in the population. This question might repay investigation with particular attention to any possible effect of nuclear constitution on heat resistance.

Fungi differ widely in their tolerance to subzero temperatures (Cole and Fergus, 1956; Faull, 1930; Flor, 1954; Harter and Zaumeyer, 1941). The rather large literature on the effect of very low temperatures, summarized in part by Luyet and Gehenio (1940), is impossible to evaluate in the light of present-day knowledge. Too often we are not informed of essential experimental conditions—the rates of freezing and thawing, the quantitative degree of injury or survival, the types of cells present in the preparation, and so on. About the only generalization that appears safe is that spores frozen in water or at high moisture content are more susceptible to freezing injury than are dry spores. Repeated freezing and thawing under moist conditions appear to be particularly damaging.

We touch here on a general biological problem, the nature of freezing injury. It is believed that in slow freezing, the most probable situation in reported experiments on fungi, the injurious factor is withdrawal of water and dehydration of the cell (Meryman, 1956). However, the possibility of internal formation of ice crystals and consequent mechanical disruption of cell structure cannot be disregarded. Finally, the experiments of Mazur (1956) show that the viability of spores of *Aspergillus* *flavus* frozen at —70 to —75° C. depends primarily on the rate of warming; the slower the return to 0°, the less the survival (Fig. 1). These experiments demonstrate that there is much to be learned about the lethality of subzero temperature and that the fungi may be suitable material for investigation.

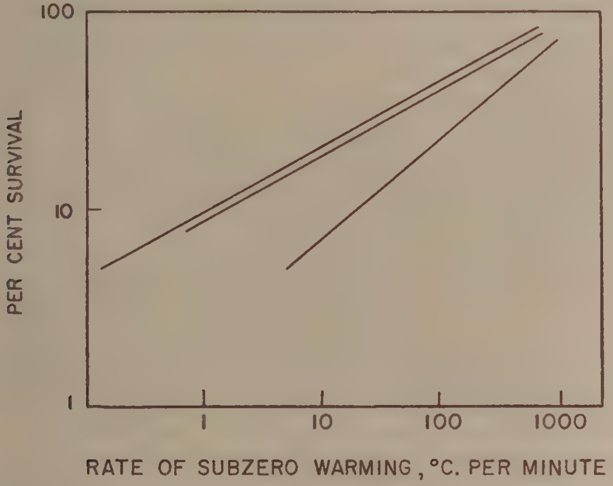

Fig. 1. The influence of the rate of warming on survival of conidia of *Aspergillus flavus* frozen at —70° C. Each curve represents a separate experiment. Redrawn from Mazur (1956), by permission of the author and of the Rockefeller Institute for Medical Research.

C. *Preservation of Spores*

In order to avoid labor in the maintenance of stock collections and to minimize genetic changes during long continued cultivation, it is desirable to find ways of preserving material for long periods of time in a dormant but viable condition. It must be said at the outset that no universally satisfactory method, applicable to all fungi, has been found.

One method has the advantage of simplicity; the fungus is transferred to sterile soil and allowed to grow and sporulate until the soil dries out. This method is useful for some fungi but not, unfortunately, for others (Atkinson, 1954), and cannot be relied upon in general.

Lyophilization (freeze-drying) of spores suspended in serum, dextran, or gelatin is more often successful, but again it is not universally efficacious (Haskins and Anastasiou, 1953; Meyer, 1955). Simple vacuum drying without freezing is often more effective, especially if the suspending medium is omitted and dry spores are used (Graham, 1956; Rhodes, 1950). Survival is also better if the spores are allowed to rehydrate slowly before being put on a germination medium (Graham, 1956; Sharp and Smith, 1957).

A study of three saprophytic fungi, all of which suffer 90% losses in viability when subjected to the standard freeze-drying procedure, indicates that rapid spray drying from a serum suspension causes less damage; the point is made that in lyophilization the rate of drying is probably too low (Mazur and Weston, 1956).

IV. SPORE NUTRITION AND METABOLISM

A. *Permeability*

Since permeability is perhaps the least understood phenomenon of biology, it is not surprising that our data on the entrance of materials into spores is fragmentary and difficult to explain. It is easy to observe in respiration studies, for example, that sugars, alcohols, organic acids, and other substrates enter the spore and are metabolized. Rubidium—and by inference potassium—is accumulated against a concentration gradient by germinated conidia of *Neurospora crassa;* limited evidence suggests that the transfer requires metabolic energy (Lester and Hechter, 1958). Toxicity of azide and fluoride is maximal at low pH (Sussman *et al.*, 1958); as in other organisms, this finding implies that un-ionized compounds enter more rapidly than ionized.

Recent work on toxicants poses two quite different views of the entrance of ions. On the one hand, spores of fungi rapidly accumulate cationic toxicants, e.g., mercury and silver, when these are supplied to the spore in fungicidal amounts (Miller and McCallan, 1957; Miller *et al.*, 1953a, b). The implication is that spores are very permeable to ions; it is believed that the amounts accumulated are too large for surface adsorption to be the primary factor, and direct analysis of homogenate fractions confirms that toxicants are distributed within the cell (Owens and Miller, 1957). Although it may be objected that the apparent rapid entry of these ions is through a membrane damaged in some manner, zinc, a nontoxic ion, is taken up in the same way.

Against this view on permeability to ions stand experiments of Sussman *et al.* (1958). Toxic anions like fluoride, supplied at concentrations probably too low for quick fungicidal action, appear to penetrate slowly; toxic cations are removed from solution by an adsorption mechanism in a short time, but reach sensitive regions of the cell only after an appreciable time.

It is not possible now to decide between these different hypotheses; the experimental methods are, indeed, so different that it is not even certain that the two are contradictory.

Studies on methylene blue uptake show that the spore has electronegative binding sites and that cations compete for these sites (Sussman and Lowry, 1955); it is known that hydrogen ions somewhat antagonize the toxicity of copper (Biedermann and Müller, 1951), and that other cations depress the accumulation of copper by *Monilinia fructicola* (Marsh, 1945). Other studies have shown that lysozyme treatment changes the permeability of ascospores of *Neurospora tetrasperma* (Lowry *et al.*, 1956). In the same species, the release of ions from the cell at the time of onset of germination can be interpreted as a change in membrane permeability (Sussman, 1954).

An antibiotic of *Streptomyces* sp. causes abnormal swelling of sensitive fungus cells; it is speculated, without direct evidence, that the antibiotic prevents an active metabolic extrusion of water (Links *et al.*, 1957).

B. *Nutritional Requirements*

One of the problems of spore physiology is to determine whether a spore requires exogenous nutrients. It is not permissible simply to wash spores from a nutrient medium and test them for germination with and without added nutrients; too many nutrient materials are carried over from the medium (Lin, 1940). Much of the relevant literature is vitiated by the absence of any description of the method used to prevent contamination of the spores by nutrients. In view of the difficulty, we may speculate that many of the instances in which spore germination is "stimulated" by nutrients are in fact instances of real nutritional deficiencies partially met by contaminating substances external to the spore.

Conventionally, one would suppose that a test could be made by washing spores by filtration or centrifugation and testing the washed spores for germination with and without nutrients. Although the present author feels that this approach is justified, it must be admitted that the method is open to the objection that essential materials may be leached from the spore during the washing process. Washed spores of *Fusarium roseum* require exogenous carbon and nitrogen (Sisler and Cox, 1954), those of *Glomerella cingulata* carbon, nitrogen, phosphorus, and sulfur (Lin, 1945). At the other extreme, washed ascospores of *Neurospora tetrasperma* germinate in distilled water (Sussman, 1954).

A second method, and one less open to cavil, is to collect spores in such a way that they are not from the beginning contaminated by nutrients. Vacuum collection, when possible, is a satisfactory method, and requirements for carbon, nitrogen, and minerals can often be shown in spores collected in this way (Berry and Barnett, 1957; Lin, 1940). On the other hand, conidia of the powdery mildew fungi and urediospores of the rust fungi collected by shaking from the infected leaf, and hence presumably free of nutrients, germinate well in pure water, although nutrients may accelerate germination (Yarwood *et al.*, 1954).

Few natural vitamin requirements for spore germination are known; biotin is required by *Memnoniella echinata* (Perlman, 1951) and—for one stage of the germination process—by *Myrothecium verrucaria* (Mandels, 1955). Hypoxanthine, partially replaced by guanine, accelerates germination of spores of *Phycomyces blakesleeanus* (Robbins and Schmitt, 1945). Complex organic materials, e.g., plant extracts, crude proteins, and soil extract, often increase germination (Gottlieb, 1950), but by mechanisms that are still unknown.

In general, mutants requiring particular metabolites for growth—amino acids, vitamins, etc.—also require them for spore germination or at least for germination at a normal rate (Ryan, 1948; Shepherd, 1956; Woodward *et al.*, 1954).

These admittedly fragmentary data suggest that there is among fungi a spectrum of nutritional requirements. At one extreme, some fungi need no external nutrients; presumably, the energy necessary for germination can be obtained from endogenous reserves. Other fungi may be absolutely or only partially dependent on a particular nutrient or, in the most extreme situation, on several nutrients. An ecological advantage may be conferred on a fungus which requires external nutrients, in that spores will be "protected" from germinating in a milieu which is too impoverished to support mycelial growth. Presumably, this advantage is offset among pathogens like the rust fungi by the ability of the spore to support germ tube development and penetration of the host.

The spores of plant pathogens germinate in an infection court which often contains nutrients derived from the host. These nutrients may be normal exudates of a leaf or root (Barton, 1957; Kovacs and Szeöke, 1956; Weintraub *et al.*, 1958), or materials lost from previously infected tissue as a result of the infection (Wilson, 1937). The plant pathologist is therefore less interested in whether a spore has an absolute or relative requirement than he is in the effect of nutrients on the "inoculum potential" (Garrett, 1956), the sum of inoculum factors which determine the establishment of an infection. A hypothetical fungus may be able to germinate in distilled water if given time enough, but the required time may be so long that under field conditions successful penetration of the host is negligible in the absence of nutrients. If spore germination is accelerated by nutrients and if nutrients are normally present in the infection court, this may make the difference between success and failure of the infection process.

C. *Metabolism of Ungerminated Spores*

Spores are complete metabolic systems; under suitable conditions, therefore, it is not surprising that common enzymes known from studies on mycelial preparations are demonstrable. It would be tedious to list all known spore enzymes; it is interesting to note that some appear from limited evidence to be located at or very near the spore surface (Mandels, 1956; Zalokar and Cochrane, 1956). Urediospores of *Puccinia graminis tritici* form several hydrolytic enzymes; pectin polygalacturonase appears, from not quite conclusive data, to be formed "adaptively," i.e., to be synthesized in response to the presence of substrate in the germination medium (Van Sumere *et al.*, 1957a).

Spores are always capable of oxygen uptake so long as they are viable. Typically, a basal endogenous rate can be measured and represents the oxidation of stored materials. Provision of a respirable substrate results in accelerated oxygen uptake, although spores of some fungi may not respond to a particular substrate (McCallan *et al.*, 1954). The substrates which are used vary of course with the fungus; *Myrothecium verrucaria* spores respire actively on the common monosaccharides, sucrose, maltose, glycerol, mannitol, ethanol, several organic acids, and some amino acids (Mandels and Norton, 1948).

Oxidative assimilation is often encountered in studies of the respiration of mycelial preparations (Stout and Koffler, 1951). In this laboratory we have noted that the oxidation of acetate by spores of *Fusarium solani* conforms to the equation:

$$C_2H_4O_2 + O_2 \rightarrow (CH_2O) + CO_2 + H_2O$$

That is, oxidation is incomplete, and we assume that the "missing" acetate is assimilated at the reduction level of carbohydrate.

Fermentative activity has been relatively little studied in spores. Unpublished work in this laboratory shows vigorous alcoholic fermentation by macroconidia of *Neurospora* spp. incubated anaerobically with glucose. With *Fusarium solani* macroconidia there is no detectable anaerobic carbon dioxide formation.

Studies with isotopically labeled glucose suggest that in some fungi, spore respiration differs qualitatively from that of mycelium (Cochrane, 1957). The methods available do not, however, allow us to specify with any assurance just which pathways are operative. It is easily conceivable, in the abstract, that the onset of growth is accompanied by or even requires some change in the pathway of respiration. These problems remain for the future.

D. *Metabolism of Germinating Spores*

At least in those fungi which are independent of external sources of carbon for germination, there must be respirable substrates the oxidation of which provides energy for germination processes. Although the fat content of many spores is not unusually high (Schönborn, 1955), it may be suggested as a working hypothesis that the oxidation of fats provides the necessary energy. First, although not conclusive by itself, the respiratory quotient of *Neurospora crassa* spores is about 0.7 (Owens, 1955). Second, stainable lipids disappear during germination (Evans and Harrar, 1930; Kordes, 1923). Third, the clear analytical data of Shu *et al.*, (1954) show that the carbohydrate and protein content of urediospores of *Puccinia graminis tritici* change little during germination, but that the lipid content falls from 19.7% to 7.8% of the dry weight during the germination process. The lipid content of spores of *Aspergillus nidulans* similarly declines during germination (Shepherd, 1957).

Physiological changes during germination of spores of *Aspergillus niger* in a glucose-proline-phosphate medium have been reported in detail by Yanagita (1957). Dry weight and total nitrogen both increase but the total nitrogen as a fraction of the dry weight declines during germination. Changes in ribonucleic acid, nonprotein nitrogen, and protein nitrogen all conform to a picture consonant with current concepts of protein synthesis: the ribonucleic acid begins to increase very early in germination, after which nonprotein nitrogen rises to a peak and then declines. During this decline of nonprotein nitrogen, protein rises, suggesting that a pool of protein precursors built up in the earlier period is being converted to protein via some process in which the ribonucleic acid plays a vital role.

It is to be expected that a somewhat different sequence of events prevails in spores which are germinating without an external source of carbon. Certainly there would be no over-all increase in dry weight. Whether protein is synthesized before exogenous sources of nitrogen and carbon can be tapped is a problem worth investigation. Similarly, the fate of any organic or polymerized phosphate would be of interest; during germination of *Aspergillus nidulans* spores both of these fractions decline in amount (Shepherd, 1957).

V. TEMPERATURE AND GERMINATION

The "cardinal temperatures" for spore germination—the minimum, optimum, and maximum—have been determined for many fungi; several summaries are available (Hawker, 1950; Togashi, 1949; Wolf and Wolf, 1947). Generalization is difficult, not least because the methods used in the original determination are often inadequate. However, it does appear that some groups of fungi can be approximately characterized by their temperature preferences. The lowest temperature optima, for example, appear as a rule among the Peronosporales, although other low temperature fungi are of course known (Niemann, 1956). Species of *Aspergillus* and possibly of *Coprinus* have rather high optima. Urediospores of most rust fungi germinate best at 22° C. or lower; again, some exceptions are apparent in the summaries cited. Such convenient generalization is not always possible: reported optima for species of *Rhizopus* range from 27° to 44° C. (Weimer and Harter, 1923), and strains of *Erysiphe cichoracearum* with very different optima have been found (Yarwood *et al.*, 1954). Similarly, one cannot generalize about the smut fungi: chlamydospores of *Tilletia* spp. germinate best at about 5° C. (Baylis, 1958; Meiners, 1958), those of *Ustilago zeae* at 26–34° C. (Jones, 1923b). Fungi which can only infect their hosts in cool weather derive an obvious advantage from a low temperature optimum.

From the data collected by Togashi (1949), it is possible to estimate that the "average" optimum for plant pathogenic fungi is about 25° C. Presumably a comparable value for saprophytic fungi would be a few degrees higher; there are no satisfactory data on spore germination in the truly thermophilic fungi.

In Fig. 2 are drawn idealized response curves of three types. The type most frequently reported is roughly symmetrical—often a little skewed to the right—and has a broad optimum zone. Examples include several of the rust fungi (Campbell and Dimock, 1955; Cochrane, 1945a), *Coccomyces hiemalis*, (Keitt *et al.*, 1937), and *Ustilago* spp. (Bever, 1945). The other two types of curve shown in Fig. 2 are quite frequent; more rarely, a curve skewed to the left has been reported (Cherewick, 1944; Felton and Walker, 1946).

A certain amount of caution in the interpretation of curve shape is, however, necessary. Numerous factors affect it, and different laboratories seldom control these factors to the same degree. The most important is the time of observation: early observation yields a curve with a sharply defined optimum, but in time the germination at less favorable temperatures catches up and the optimum becomes much broader (Felton and Walker, 1946; Ling and Yang, 1944; Saccas, 1951; Wellman and McCallan, 1942).

The shape of the response curve is also affected by other environmental factors. It may be stated as a principle that the limits of germination are narrower if some other factor is nonoptimal. In specific terms, it is found that the temperature range permitting germination is narrower if spores are immature (Doran, 1922), if the pH is unfavorable (Tilford, 1936), if relative humidity or substrate moisture is nonoptimal (Groom and Panisset, 1933; Purdy and Kendrick, 1957), or if nutrients are in short supply (Gardner, 1918; Yarwood *et al.,* 1954).

In general, the temperature response of germ tube growth is more like that of mycelium than it is like that of spore germination per se; the data of Snell (1922) on *Lenzites sepiaria* are especially striking.

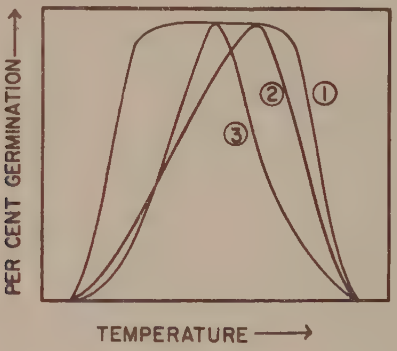

FIG. 2. Idealized types of temperature-response curves of spore germination. The distinctive features are exaggerated for emphasis. Curve 1: symmetrical with a broad optimum. Curve 2: skewed to the right. Curve 3: symmetrical with a sharp optimum.

Again, it is impossible to generalize: germ tube growth and germination may respond alike to temperature ((Frick, 1943; Gäumann, 1946; Saccas, 1951) or there may be pronounced differences (Cochrane, 1945a; Gäumann, 1946; Felton and Walker, 1946; Keitt *et al.,* 1937; Yarwood *et al.,* 1954).

So far we have considered data on the effect of temperature on the final germination, i.e., on the potential germination. If the rate of germination is the criterion, we find quite commonly and with a variety of different plant pathogenic fungi that the rate is more sensitive to moderately nonoptimal temperatures than is the final germination. That is, germination is delayed by temperatures lower or higher than the optimum, but, once germination has begun it is almost as rapid and complete at the unfavorable temperature as at the favorable (Cochrane, 1945a; Saccas, 1951; Sweet, 1941; Wellman and McCallan, 1942). Put another way, the latent period is more sensitive to an unfavorable temperature than is the final germination.

This sensitivity is paralleled by sensitivity of the early processes of spore germination to a favorable temperature. The germination of urediospores of *Phragmidium mucronatum* at 9° C. is increased by about 160% if the spores are exposed for one hour to a temperature of 18°; during this exposure at a favorable temperature there is no visible sign of germination (Cochrane, 1945b). The same phenomenon apparently occurs in *Puccinia chrysanthemi* (Campbell and Dimock, 1955); in *Phytophthora infestans* even a very short period at 40° C. greatly increases direct germination of conidiosporangia subsequently incubated at 20° (Taylor *et al.*, 1955). We may suggest, therefore, that the early metabolic reactions in spore germination have a different response to temperature than later stages.

VI. WATER AND GERMINATION

The association of water with fungus development is a matter of common observation, and the economic importance of the fungi has ensured the accumulation of a substantial body of evidence on the moisture requirements of spore germination (Clayton, 1942; Doran, 1922; Gottlieb, 1950; Siu, 1951).

Most results are reported in terms of relative humidity. It is not always realized, however, that any measurement of relative humidity is only as accurate as the temperature determination. Particularly if one is trying to maintain an atmosphere at 100% relative humidity without the presence of liquid water, temperature control is decisive; at the 100% level, even the slightest lowering of temperature must result in deposition of free water; in such experiments, the precision of control of temperature should be of the order of ± 0.01° C. Systems for the control of humidity have been described (Clayton, 1942; Cochrane, 1945a; Delp, 1954); the author's experience indicates that a static system, in which spores are incubated in a closed vessel over a humidity-regulating solution, is preferable in accuracy and convenience to systems which depend on controlling the moisture in a moving air stream.

It is often of interest to measure the germination of spores on solid substrates—agar, grain, textiles, etc.—which themselves take up water from the atmosphere. Under such conditions, germination is often possible at relative humidities which would not permit germination on a nonabsorptive medium like glass (Armolik and Dickson, 1956; Block, 1953; Groom and Panisset, 1933). It is reasonable that such findings be interpreted as indicating that the substrate has a greater water-absorbing capacity than the spore but that in some way the spore is able to utilize this water. We have thus a paradox, the spore apparently getting water from a substrate which has a higher water-absorbing capacity than it does. Only further study, with methods more subtle than those heretofore used, will resolve the difficulty.

Fungus spores which are able to germinate at rather low humidities are correspondingly able to germinate in media of high osmotic pressure (Armolik and Dickson, 1956).

Temperature and humidity requirements are so interrelated that it is somewhat artificial to speak of an optimum for either without specifying the other. In general, as the temperature is raised, the requirement for water becomes more stringent, i.e., the relative humidity required for best germination rises (Bonner, 1948; Delp, 1954). We have already mentioned that the apparent temperature optimum is affected by the relative humidity.

The fungi can be arranged along a scale of humidity requirements. At one end of the scale we find the powdery mildew fungi, some of which produce spores able to germinate at zero relative humidity. At the other extreme, a number of fungi appear to require liquid water for germination. Let us consider first the intermediate forms.

Several fungi, including species of *Aspergillus* and *Penicillium*, form spores which germinate at as low a relative humidity as 75 to 80% (Armolik and Dickson, 1956; Bonner, 1948; Heintzeler, 1939). This value seems to be the practical low limit for fungi apart from the powdery mildew fungi. Somewhat more form spores which germinate at relative humidities of 90 to 95%; some examples are *Ustilago* spp. (Clayton, 1942), *Botrytis cinerea* (Rippel, 1933), *Fomes annosus* (Rishbeth, 1951), and *Verticillium albo-atrum* (Schneider, 1954). *Venturia inaequalis* ascospores and conidia require a yet higher relative humidity but still germinate in the absence of liqud water (Clayton, 1942).

The top of the scale is occupied by those fungi which require liquid water for substantial spore germination. As mentioned earlier, determination of this kind of requirement demands extremely accurate control of temperature; we must reject all reports in which accurate temperature control is not specified and germination in the absence of free water is claimed. A requirement for liquid water has been reported for *Endoconidiophora fagacearum* (Cole and Fergus, 1956), *Sclerotinia* spp. (Clayton, 1942), and the asexual spores of members of the *Peronosporales* (Doran, 1922; Hyre and Cox, 1953).

The bulk of the evidence—some, to be sure, based on inadequate methods—indicates that the urediospores, and indeed all spore forms, of the rust fungi require liquid water for germination (Cochrane, 1945a; MacLachlan, 1936; Yarwood, 1939; Zimmerman, 1925). However, the work of Clayton (1942), in which adequate methods were employed, stands in contradiction; he found that urediospores of *Puccinia coronata* and *P. graminis* germinate at 100% relative humidity in the absence of free water. A reexamination of this whole question would be of considerable interest.

As briefly noted above, the conidia of some of the powdery mildew fungi are distinctive in that they germinate at very low relative humidity, even over a desiccant (Brodie, 1945; Cherewick, 1944; Delp, 1954). Correspondingly, it is a matter of general knowledge that some of the powdery mildew diseases are favored in nature by relatively dry conditions.

The virtually unique property of germination at zero relative humidity confers on the powdery mildew fungi considerable theoretical importance. In a series of papers, Yarwood (1936b, 1950, 1952) has developed the fundamental idea that the ability of these spores to germinate at low humidity is a function of their high water content. Two different methods of determining water content both assign a value of about 70% water to the conidia of *Erysiphe polygoni;* density data are in agreement. By contrast, the spores of other fungi, e.g., *Penicillium digitatum* and *Monilinia fructicola*, are much lower in water and are probably in a relatively simple equilibrium with the environment. The observed decrease in volume of conidia of the powdery mildew fungi during germination in dry air supports Yarwood's hypothesis.

The hypothesis has been broadened by Yarwood (1950) in the proposition that fungus spores of the usual type, i.e., those with a low water content in hygroscopic equilibrium with the environment, must absorb water to about the level of that in the powdery mildew conidia, 70%, before germination is possible. This of course explains the swelling of spores so frequently reported (p. 170), but leaves unexplained the situation in those few spores of normal water content which apparently do not swell prior to germination.

Although there are conflicting data and an alternative explanation has been suggested for the behavior of the spores of powdery mildew fungi (Brodie, 1945), the proposal just outlined appears to be the most promising general explanation of humidity relations. Some problems of course remain; for example, the conidia burst in pure water rather soon after immersion (Delp, 1954), implying a rapid entrance of water. On the other hand, the observed maintenance of a high water content even over desiccants, and direct measurements of water loss (Yarwood, 1950), both suggest that the outward movement of water from the interior of the spore is slow.

VII. pH AND GERMINATION

Spores of most fungi germinate best at pH 4.5 to 6.5, with extreme limits at, generally speaking, pH 3 and 8. A number of examples may be cited in support of this rough generalization: spores of Myxomycetes

(Smart, 1937), chlamydospores of *Urocystis tritici* (Noble, 1924), urediospores of rust fungi (Allen, 1955), and ascospores (Butler, 1956). Naturally it is not difficult to find fungi which do not fit the generalization; some have rather acid optima (Tilford, 1936), others germinate best at higher pH values (Cole and Fergus, 1956; Webb, 1921), and still others have very broad optimum zones (Chowdhury, 1946; Fergus, 1957; Webb, 1921).

No doubt some of these differences reflect differences in experimental method rather than fundamental biological relations. In studies of spore germination and pH it is desirable that a buffer be used; unfortunately, the type of buffer chosen may influence the shape and limits of the response curve (Sussman, 1954). The form of the pH response curve is also affected by the previous history of the spore population (Allen, 1955) and by nutrient materials which may be added to the germination medium (Butler, 1956; Webb, 1921).

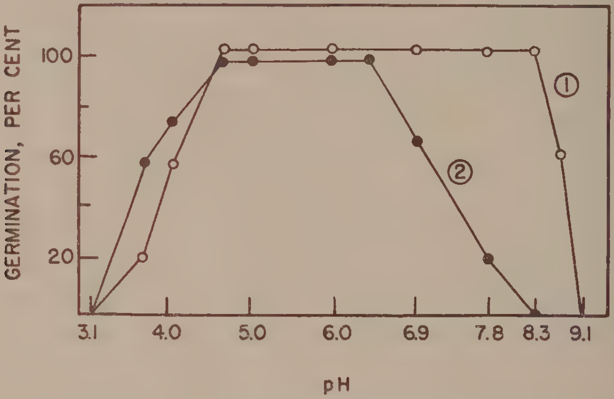

FIG. 3. The influence of pH, in a buffered medium, on the germination of urediospores (Curve 1) and aeciospores (Curve 2) of *Puccinia sorghi*. Redrawn from Le Roux and Dickson (1957), by permission of the authors and of *Phytopathology*.

It must be remembered that pH is not a unitary factor and that the mechanism of pH action may be different at different concentrations of hydrogen ion. Thus, one limb of a pH-germination curve may be generated by acid inactivation of a surface enzyme, the other by a totally different mechanism, e.g., precipitation of essential metals. The entrance of nutrient materials, if they are required for germination, may be decisively affected by pH, especially if the compounds are ionized at biological pH values. In the poorly buffered media so often used, it is possible—although it has not yet been reported—that metabolic activities of the germinating spore modify the pH; the effect of such modification would be in general to permit germination over a range of pH values somewhat wider than the range in buffered media.

A double pH maximum for spore germination has been reported (Forbes, 1939; Tilford, 1936; Webb, 1921). No such case has been thoroughly analyzed; studies on mycelial growth of *Coprinus* spp., however indicate that a double optimum merely reflects pH-dependent unavailability of one or more inorganic elements and is eliminated in a medium containing available iron, zinc, and calcium (L. Fries, 1956).

The pH requirements of the different spore forms of *Puccinia sorghi* are somewhat different (Fig. 3).

It seems unlikely that pH is often the limiting factor for spore germination under natural conditions either in the soil or on exposed plant parts. Possibly in a few unusual environments—very acid soil, acid plant juices, etc.—it may become of some importance in affecting the occurrence of plant disease.

VIII. Light and Germination

A. *Visible Light*

In general, as Gottlieb (1950) points out, visible light (roughly, 400 to 800 mμ wavelength) has little effect on spore germination of most fungi unless the intensity is so high that heating becomes significant. Light of this range is of course photochemically active but only under favorable conditions; the energy per einstein, i.e., per mole of photons, is 35,500 to 71,000 calories, sufficient to drive a number of chemical reactions if conditions are favorable but not nearly so energetic as the shorter wavelengths of the ultraviolet region. Consequently, it is to be expected that ultraviolet light will exert biological effects more uniformly than will visible light, but that in the presence of a receptor and a sufficiently labile system visible light too will influence biological systems under some circumstances.

The first of these circumstances is photodynamic action. In artificial systems, a dye absorbs light and in the presence of oxygen (usually) the energy absorbed is used to drive chemical reactions, presumably photooxidations. Photodynamic action has been studied less in the fungi than in other organisms; conidia of *Penicillium notatum* which have been stained vitally with erythrosin are thereby rendered sensitive, as estimated by mutagenesis, to visible light (Kaplan, 1950). Ascospores of *Neurospora crassa* are sensitized similarly by eosin (Döring, 1938), urediospores of *Puccinia graminis tritici* by Congo red (Dillon Weston, 1932a).

A natural photodynamic action of chlorophyll has been discovered in a study of carotenoid-deficient mutants of the photosynthetic bacterium *Rhodopseudomonas spheroides* (Stanier and Cohen-Bazire, 1957). It is suggested that in the wild type—and, indeed, in all photosynthetic organisms—carotenoids protect against this photodynamic action, possibly being themselves oxidized to epoxides. This leads further to the possibility that carotenoids in general protect against photooxidations; thus, Stanier and Cohen-Bazire suggest that the carotenoids of fungus spores, and for that matter of mycelium, protect against damage from light. Chlorophyll is not of course present in fungi, but other porphyrins may take its place; or, still other pigments may be involved. *In vitro* studies have shown, for example, that riboflavin can act photodynamically (Galston and Baker, 1949).

A second, and more familiar, effect of visible light is the inhibition of spore germination. This is especially well documented in the rust fungi; the literature is reviewed briefly by Cochrane (1945c). The striking aspect of this inhibition is that experiments with filters indicate that the longer wavelengths—red, orange, and yellow light—are the effective wavelengths, and that the higher energy radiations in the blue regions are ineffective (Dillon Weston, 1932a). This rather surprising conclusion is borne out by studies on the inhibition by light of germination of the conidia of *Erysiphe graminis tritici*, which are of course not obviously pigmented (Pratt, 1944). A thorough quantitative study of this problem, including a complete action spectrum with high resolution and a determination of the temperature coefficient, would be of great interest.

Dry spores are less susceptible than wet to light inhibition (Cochrane, 1945c); this is also true of ultraviolet inhibition (Dillon Weston, 1931). Presumably under these conditions, some growth processes have begun and sensitivity is thereby increased; partially germinated spores are more sensitive than ungerminated (Dillon Weston, 1932b; Pratt, 1944).

Visible light causes a bending away of the germ tubes of germinating urediospores of some rust fungi (Forbes, 1939). This negative phototropism suggests that the proximal wall of the germ tube is accelerated in its extension, i.e., a stimulatory rather than an inhibitory action, but the mechanism is unknown. The most effective region of the visible spectrum is the blue-violet.

Gottlieb (1950) reviews the early literature on light acceleration of spore germination, most of which is suggestive only. However, the more recent work of Ziegler (1948) on spore germination in the Saprolegniales, and especially the data of Hebert and Kelman (1958) on *Physoderma maydis* of the Chytridiales, definitely establish a role of light energy in spore germination. Some of the data on *P. maydis* are shown in Fig. 4. Again, it is the shorter wavelengths which are effective and, consistent with a possible role of photooxidations, the light effect is exerted only in the presence of oxygen. The light response of spore germination of *Erysiphe polygoni* is conditioned by the time of removal of the conidia from the host plant (Yarwood, 1936a).

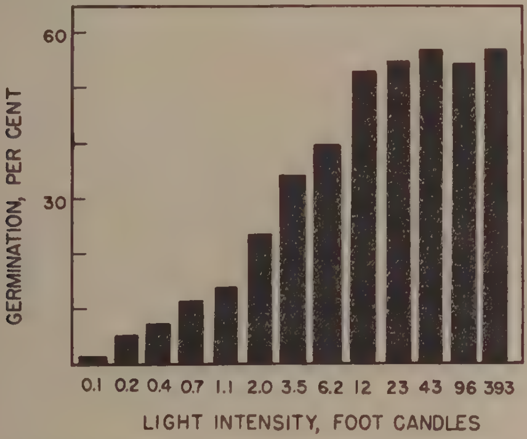

FIG. 4. The germination of resting sporangia of *Physoderma maydis* as a function of light intensity. Redrawn from Hebert and Kelman (1958), by permission of the authors and of *Phytopathology.*

Finally, we may briefly mention as an effect of visible light the phenomenon of photoreactivation, reviewed by Dulbecco (1955). This may be defined as the partial reversal of lethal, mutagenic, or other effects of ultraviolet radiation by subsequent exposure, within a short time, to visible light. An action spectrum has not been reported for fungi; for *Streptomyces griseus,* photoreactivation is maximal at 436 mμ (Kelner, 1951).

Diurnal variations in germ tube growth and in germinability of conidia of *Erysiphe* spp. (Yarwood, 1936a; Yarwood and Cohen, 1949) cannot as yet be ascribed to any direct effect of light, since the conidia are of necessity grown on a host plant and indirect effects cannot be excluded.

These various effects of visible light—inhibition of germination, acceleration of germination, germ tube tropisms, and photoreactivation—cannot as yet be subsumed under one theory. For each, we need to know the action spectrum, the temperature coefficient, and the effect of such modifying factors as oxygen tension. Only after these facts are known will it be possible to investigate the nature of the photoreceptor and of the primary photochemical reaction.

B. *Ultraviolet Radiation*

The exposure of fungi to ultraviolet radiation has two main effects, mutation and death (Pomper and Atwood, 1955); conidia of *Erysiphe graminis* lose the ability to initiate infection faster than the ability to germinate (Buxton *et al.*, 1957). In general, the response of fungi is not qualitatively different from that of other microorganisms; most published action spectra, for example, show a maximum effect at about 265 mμ, indicating that absorption by nucleoprotein is the primary event. More problems are raised by the shape of the survival curve—sigmoidal in many fungi but exponential in some. The most probable explanation for these differences is in the number of nuclei per spore: irradiation of uninucleate microspores of *Neurospora crassa* yields an exponential survival curve, but exposure of multinucleate macroconidia generates a family of sigmoidal curves in which the number of nuclei per spore determines the shape of the curve (Norman, 1951, 1954). That is, conidial inactivation is the result of nuclear inactivation, and a number of hits is required to inactivate a multinucleate spore. Correspondingly, the kinetics of photoreactivation show that reactivation of a single nucleus is sufficient to reactivate the spore.

Although nuclear inactivation seems therefore to be the determining event in lethality, it does not follow that death can be equated with lethal mutation. Norman (1951) argues persuasively for a dual effect: lethal mutation and nongenetic damage to the nucleus.

The effect of ultraviolet radiation on fungi is modified by environmental factors to a degree inconsistent with a simple target theory of action. Mutagenesis by ultraviolet, for example, is increased by pretreatment with heat, 2, 4-dinitrophenol, or nitrogen mustard (Pomper and Atwood, 1955). Postirradiation treatment with high hydrostatic pressure reduces the recovery of mutants from irradiated *Neurospora crassa* populations, suggesting that the primary action of ultraviolet is the formation of a semistable intermediate which may either return to its original state or be converted to a new, mutant state (McElroy and Swanson, 1951). Alternatively, of course, the influence of environmental factors may be construed as support for a theory of an indirect action of radiation on the spore, possibly through a photochemical reaction in the medium. However, it appears that at the biologically effective wavelengths peroxide formation is not sufficient to explain the action of ultraviolet (Pomper and Atwood, 1955).

Photoreactivation, discussed in the previous section, virtually compels us to imagine one or more intermediate steps between absorption of ultraviolet and the final biological effect, and to hypothesize that all effects of ultraviolet have at least one stage in common, since all are subject to photoreversal.

IX. Effects of Oxygen and Carbon Dioxide on Germination

Ideally, experimental studies on the oxygen requirement of spore germination should include (1) a study of the response of spores to graded and accurately measured oxygen pressures, and (2) proof that the spores which do not germinate at a given oxygen pressure are still viable at the end of the observation period. As pointed out by Gottlieb (1950), it is not possible to conclude simply from a deleterious effect of submergence on germination that the mechanism responsible is an oxygen deficit; too many other factors, especially the accumulation of carbon dioxide and of toxic metabolites, enter into the picture.

In general and with few exceptions, spores of the fungi do not germinate under complete anaerobiosis; examples include urediospores of rust fungi (Allen, 1955; Hart, 1926), ascospores of *Neurospora crassa* (Goddard, 1935), and chlamydospores of smut fungi (Jones, 1923a, Platz, 1928). Conidia of *Erysiphe graminis* are reported by Domsch (1954) to germinate, although poorly, in a nitrogen atmosphere, but other studies on the powdery mildew fungi indicate that anaerobiosis prevents spore germination (Brodie and Neufeld, 1942).

Conidiosporangia of *Phytophthora* spp. germinate—by swarmspore formation only—under anaerobiosis, but spores of other species of the Peronosporales fail completely to germinate under these conditions (Uppal, 1926). Even in the Mucorales, many species of which appear to have a low oxygen requirement, germination is apparently not possible under completely anaerobic conditions (Wood-Baker, 1955).

We have relatively little information on the optimum oxygen pressure for spore germination. Quantitative studies on three different fungi —*Puccinia graminis tritici* (Allen, 1955), *Botrytis cinerea* (Brown, 1922), and *Ustilago zeae* (Platz, 1928)—agree that an oxygen pressure of about 30 to 38 mm. is sufficient for spore germination.

Spore germination and respiration of *Aspergillus niger* are accelerated by carbon dioxide (Yanagita, 1957); we may speculate that the role of carbon dioxide in fungal metabolism is as a precursor of essential amino acids. The requirement is specific: *Ustilago maydis* germinates only negligibly but *Physoderma maydis* germinates normally in an atmosphere free of carbon dioxide (Hebert and Kelman, 1958). The essentiality of carbon dioxide is of course difficult to demonstrate in complex media, and impossible to detect once metabolic production of carbon dioxide has begun. Inhibition by carbon dioxide has been demonstrated (Gottlieb, 1950), but the mechanism has not been worked out; it is possible that specific factors operate when the inhibitory level is low—1% or so—and that inhibition by high carbon dioxide pressure is indirect, perhaps through effects on acidity or on oxygen supply.

X. Some Effects of the Biological Environment

Mucilaginous or other matrices in which spores are produced are believed to have some effect in prolonging their life under adverse conditions, particularly during periods of desiccation (Gottlieb, 1950). The "honey dew" of *Claviceps purpurea,* suitably diluted, promotes the germination of the spores which are produced within it (Garay, 1956).

It has been known for some time that spores germinate poorly if the suspension is too dense. In the past, the tendency was to ascribe this, without investigation, to oxygen lack or to excessive carbon dioxide. The phenomenon, now termed self-inhibition, has been studied critically in several rusts (Allen, 1955; Yarwood, 1954, 1956a, b), both *in vitro* and on host leaves. The substance (or substances) is volatile, heat stable, adsorbed or otherwise removed from solution by glass surfaces, and more effective at high than at low pH. The material is produced by urediospores under aerobic conditions. There is some evidence, not yet conclusive, that urediospores of *Puccinia graminis tritici* produce 2-methyl-butene-2 (trimethylethylene), and it has been suggested that this may be the active inhibitor (Forsyth, 1955).

Possibly analogous materials are produced by *Aspergillus niger* (Krishnan *et al.*, 1954) and by *Coccomyces hiemalis* (Keitt *et al.*, 1937); these appear, however, to be present in the spore at the time of its liberation and to be removable by washing.

Self-inhibition has the obvious advantage that spores would tend not to germinate before dispersal, while crowded within the fruiting structure.

Discovery of self-inhibition among the rust fungi may explain the earlier finding (Parker-Rhodes, 1939) that extracts of rusted leaves inhibit urediospore germination; this was originally interpreted as some form of acquired immunity. However, the material in the extracts was reported to be species-specific, whereas the volatile inhibitor appears to be the same in different rust fungi (Yarwood, 1956b).

Pelargonaldehyde (*n*-nonanal) is found in extracts of urediospores of *Puccinia graminis tritici* which have been allowed to autolyze; it is stimulatory to urediospore germination (French and Weintraub, 1957). Paper chromatography of urediospores of this rust fungus demonstrates a number of phenolic compounds, e.g., coumarin, vanillic acid, and protocatechuic acid, and several of these are stimulatory to germination (Van Sumere *et al.*, 1957b). This stimulation of germination may, it is suggested, result from counteraction of a self-inhibitor.

Two intraspecific reactions of adjacent germ tubes should be mentioned; it will be obvious that the two may be in fact identical. First, the germ tubes of urediospores of *Uromyces phaseoli* grow somewhat faster when the spores are sown thickly than when they are sown thinly on an agar surface (Yarwood, 1956a). Second, the observation has been made repeatedly—the literature is reviewed by Stadler (1952)—that in dense suspensions germ tubes exhibit a negative tropism toward each other. In *Rhizopus nigricans*, germ tubes form on the side of the spore distant from other spores and bend away from other germ tubes during growth. The phenomenon occurs also in species of *Mucor*, *Aspergillus*, and *Penicillium*.

A negative tropism can be interpreted as resulting from an increased rate of extension of the cell wall on the side proximal to the stimulus. It is for this reason that it was suggested above that a single growth factor may account both for self-stimulation of germ tube length and for the tropic response. However, Stadler (1952) presents some, although by no means conclusive, evidence that the negative tropism is not a reaction to a growth factor; instead, he postulates an unstable inhibitor which causes a thickening of the spore or germ tube wall and directs growth, therefore, away from the stimulus. It is to be hoped that further study of this problem will be undertaken.

A factor in normal soils which inhibits germination of spores of many different fungi has received considerable attention; the literature has been reviewed by Hessayon (1953), Jackson (1958), and Garrett (1956). The nature of the substance is completely unknown; possibly, it should be classed as an antibiotic. It occurs in the upper layers only of the soil and is destroyed by autoclaving. Even rather small amounts of known nutrients or of various plant materials antagonize the inhibition and permit spore germination. More important, the effect of the inhibitor on *Helminthosporium sativum* is reduced in the presence of susceptible plants. Ecologically, this may therefore represent still another mechanism by which spores are "protected" from germinating until they are in an environment which will, by reason of the presence of nutrients or some host plants, support further growth. The finding that plant materials antagonize the factor has led Chinn and Ledingham (1957) to suggest a control measure for *Helminthosporium sativum:* if, in the absence of a suitable host, plant materials which overcome the inhibition are added to soil, the spores will germinate but fail to develop, since this species can not maintain itself saprophytically. In effect, this procedure would reduce the inoculum potential of the pathogen.

It is possible that the "rhizosphere effect"—the increase in growth of microorganisms in the region closely adjacent to plant roots—may be related to the soil inhibitory factor and may represent an antagonism or counteraction of it (Jackson, 1957). A somewhat wider extension of this principle is suggested by the stimulatory effect of onion seedling roots on germination of the sclerotia of *Sclerotium cepivorum* (Coley-Smith and Hickman, 1957).

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CHAPTER 6

The Mechanical Ability to Breach the Host Barriers

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I. INTRODUCTION

The phrase "the mechanical ability to breach the host barriers" implies the use of force by an invading organism during penetration. Under natural conditions it is most difficult to prove whether or not barriers have been altered by chemical action in advance of what appears to be mechanical penetration. Rigorous proof of purely mechanical activity by the parasite has only been obtained by observing the penetration of artificial membranes resistant to chemical secretions. There is, however, good evidence that there are natural barriers which prevent the passage of a pathogen by physical means other than by opposing force. As an example, the hydrophobic surface of the intercellular spaces of plant tissues may provide a barrier to the rapid spread of bacteria by preventing the formation of a continuous water film. Consequently, the definition of a mechanical barrier is taken here to be "a barrier which exerts a physical rather than a chemical effect in preventing the passage of a potential pathogen." Only the effects of such a barrier may be visible.

The spread of pathogens in the host ranges from systemic infections, organ- or tissue-limited infections, to quite local attacks. In truly systemic diseases, the pathogen is usually latent until some nutritional and developmental change occurs in the host (e.g., *Epichloe typhina*) (Sampson, 1933). In the latent condition, *E. typhina* remains as a sparse intercellular mycelium spread throughout all but the roots of the host. In this condition there is no real barrier to such an intercellular pathogen, and its demands on the host are slight and cause no apparent defense reactions. In the reproductive stage, the hyphae not only force their way between the epidermal cells to the outside, but also invade the parenchyma and xylem cells. This alteration in behavior is due to some nutritional change in the host together with the necessity for a higher plane of nutrition for the reproduction of the fungus.

Macroscopically, internal barriers may often be identified from their characteristic effect on the symptoms (the striping of leaves in many cereal diseases, the angular leaf spots in dicotyledonous plants), and there are also instances of selective cortical or vascular invasions. Lack of penetrative force, or the absence of an effective cellulytic or lignolytic enzyme system, may prevent a pathogen from passing through fibrous or vascular tissues. This appears to be commonly found in organ- or tissue-limited infections and may also explain the limitation of infections to flower buds, root tips, or ovaries where only the young stage is susceptible, although this has still to be proved.

Infection may be localized by the formation of suberized layers as a response to the pathogen. This has been well illustrated by Cunningham (1928), but he has also described examples of localized infection, with no visible barrier to the spread of the pathogen. Paddock (1953) has described a similar failure of an infection to develop following successful entry.

The four groups of pathogens included here are the viruses, nematodes, bacteria, and fungi. Each will be mentioned when the relevant barrier is discussed. Viruses are distinct from the other three in their purely intraprotoplasmic development; and they probably only enter a host through wounds. Nematodes differ from the others by virtue of their relatively complex structure, for whose development a higher plane of nutrition is clearly essential. They possess a stylet which is self-evidently a means of penetration by force. Bacterial action on host cell walls is entirely chemical. Their rapid spread is dependent on a low viscosity of the surrounding liquid. Such a liquid has a low angle of contact with the cell walls, and thereby can spread easily along the intercellular spaces. The fungi, about which there is most information, have something in common with both nematodes and bacteria in their methods of breaching host barriers.

II. The Mechanism of Penetration by Force

Whether a pathogen is attracted to its host or not has long been a subject of study and speculation. There is no evidence as yet to suggest the existence of any such tropism in viruses, but motile bacterial species have been shown to react chemotropically to a gradient of chemical substances. There have also been suggestions that root diffusates may have a tropic effect on nematodes in addition to effects on their hatching and emergence (Chitwood and Oteiga, 1952). But the evidence is not entirely convincing. However, Christie (1932), Linford (1942), and others have described nematode behavior which would suggest a positive reaction to a thigmotropic stimulus, even if such was not their actual conclusion. The author, using artificial membranes, has demonstrated that thigmotropism is an important, if not the major, factor in inducing adhesion to the host (Dickinson, 1959). It has been claimed that both positive and negative chemotropism can be demonstrated in fungi. The importance of positive chemotropism in host-pathogen relationships results from the attraction of the latter up a "chemical gradient" to its source in a suitable substrate.

In the fungi, the two main tropisms are hydrotropism and thigmotropism. Positive hydrotropism or growth along an increasing relative humidity gradient was demonstrated in *Puccinia glumarum* by Balls (1905) and in *Cladosporium* by Bond (1938). It is now commonly accepted that a contact tropism is involved in the penetration of many barriers, but what is not yet understood is its nature in host-pathogen relations, more particularly when the pathogen is an obligate parasite.

Hawkins and Harvey (1919) have attempted to relate the force required to penetrate to the osmotic pressure in the fungal hyphae. They clearly envisaged what is now called "elongation growth" (Frey-Wyssling, 1957). Brown and Harvey (1927) considered that the intermolecular forces active in the process of growth by intussusception (i.e., interpolation of new macromolecules) might be the means of exerting force at the earliest stages of penetration, the indentation of the cuticle. Later stages might well be due to elongation growth. From the microscopic appearance of penetration many authors deduced the use of force by fungal pathogens. In their description of the penetration of internal parenchyma walls by force, Leach (1923) and Paddock (1953) have adduced the bending of the hypha in the cell as evidence of the use of mechanical force. This is, however, not valid evidence, as it could be interpreted, as Nusbaum and Keitt (1938) did in entrance of apple scab, as being due to elongation growth of the older part of a hypha, once its base and apex were securely fastened. Hawkins and Harvey (1919), and Paddock (1953) have described failure to penetrate with a low angle of approach. This does appear to be possible, but it is negative evidence that force is required for penetration. A more satisfactory interpretation would be failure to get adhesion with such a low angle of approach and along a cellulose wall. The only really valid evidence of mechanical force being used before penetration of internal walls is where Leach (1923) and Paddock (1953) have described the bending outward of a wall by a hyphal tip with a high angle of approach. This could only be due to force exerted by the growing apex of the hypha in pushing against the wall.

In discussing the breaching of host barriers, it is essential to distinguish between that due solely to mechanical force and that due to chemical softening of the barrier, which the pathogen can then breach by the expenditure of little or no physical force. The three main softening or solubilizing systems found in the fungi are the pectolytic, cellulytic, and lignolytic systems. The latter two systems are those found in wood rotting fungi. When only the cellulytic system is present, then the rotted timber is brown, when only the lignolytic is present it is white.

The breaching of the cell wall barrier is intimately connected with its structure. The external layer, lining the intercellular spaces and between the cells (the middle lamella), is made up of pectates (largely calcium salts). Toward the lumen is the primary wall, with inward an increasing ratio of cellulose to pectic substances. The secondary wall lies within what in most parenchymatous cells is probably a pure cellulose layer. The cellulose in the walls is in the form of a crystalline microfibrillar (micellar) system, between the interstices of which may be deposited amorphous cellulose, cutin, suberin, lignin, etc.

The ratio of crystalline to amorphous cellulose has an important bearing on its strength and elasticity. This ratio, as well as the crystalline orientation, may vary. Certainly, in general, the crystalline orientation becomes more nearly parallel nearer the lumen. Impregnation with cutin or suberin in the amorphous matrix renders a wall largely impervious to water, while impregnation with lignin increases the tensile strength.

When a pathogen passes through a host cell wall, whether external or internal, its structure undoubtedly affects the type of penetration. This will vary according to the ability of the pathogen's enzyme to affect the whole, a part, or none of the layers of wall material. Histological examination, together with a knowledge of the enzyme systems, can add considerably to the understanding of the processes involved in penetration although its results may not be easily interpreted.

The following interpretation of passage by fungi and nematodes is mainly concerned with penetration of the external host walls. Penetration of the internal walls has similar features, but does not as a rule show—due to the absence of the cuticle—so much variation.

In the fungi, after the hyphae have made contact with a suitable surface, some growth in close contact with it takes place. This is the start of what will become the appressorium. The amount of growth of hyphae in close contact with the host cell wall would appear to vary according to the host-pathogen complex, but more particularly with the angle of approach. With low angles of approach, a greater length of contact growth occurs but there may be no penetration. In fact, such hyphae have been observed to grow away from the surface (Leach, 1923). With high angles of approach, the area of contact growth may be quite small but, other things being equal, penetration takes place. Examples in which no appressorium has been observed will be discussed later. Originally, the close adhesion resulting from contact growth was thought to be due to the mucilaginous tip of the hyphae. Following the report of good adhesion of hyphal tips without mucilage, together with the cytological evidence of close adhesion between host and pathogen walls, it is now believed that the adhesion is due to the intermolecular forces between two surfaces in close contact (Blackman, 1924), the contact being particularly close since one surface has grown in contact with and over the preexisting surface.

Following contact and a halt to the further growth of the hypha, the "so-called" appressorium is formed. This is a part of the original hypha with an increased diameter and its length dependent upon the angle of approach. In some species, e.g., *Pellicularia filamentosa*, multiple appressoria are formed (Kerr and Flentje, 1957). By increase in diameter, an increase in the adherent area is obtained. With secure fastening at either end of the hypha, elongation growth may still continue, and so "looping of the hypha" [as Nusbaum and Keitt (1938) named it] will then occur. Others have also illustrated the same phenomenon (Blackman and Welsford, 1916; Dey, 1919).

The whole of the appressorial wall is now nonextendable, and in due course a new growing point will normally arise in the adherent area. Why this should form as a rule within the adherent area is not yet known. For its formation to take place, a loosening of the original bonding of the crystalline macromolecules of the wall must take place, thus making that small part of the fungal wall plastic. In experiments using impenetrable membranes and the fungi *Puccinia triticina* and *Erysiphe graminis*, new growing points have been observed appearing on the opposite side of the appressorium when the adhesion has been very good. On some surfaces, *Puccinia triticina* has been observed producing three growing points equidistant from one another at the base of the cone of the original hyphal tip. This is similar, except for the activating stimulus, to that observed by Robertson (1958) in *Fusarium*.

Only five descriptions or illustrations (Hassellring, 1906; Waterhouse, 1921; Nusbaum, 1935; D'Oliveira, 1938; Dickinson, 1949a) have been found describing unsuccessful penetration. Hassellring (1906) and D'Oliveira (1938) illustrated growth from within the center of a "pore" in the adherent area of the hypha and out from between the appressorium and the glass surface; presumably adhesion had failed. Nusbaum (1935) drew examples where adhesion had failed on a host leaf surface, and the young growing point continued as a fine "penetration" tube arising within the pore. Many other observations have been made on the presence of "pores," and it has been noticed that their diameter was greater than that of the penetration tube. Nusbaum and Keitt (1938) describing the entry of *Venturia inaequalis* into apple leaves, comment

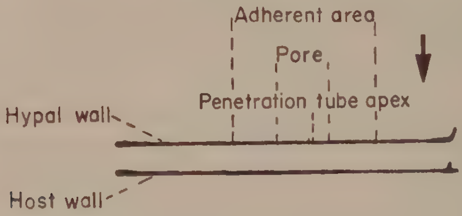


FIG. 1. Complete adhesion between fungus and host wall. (Original.)

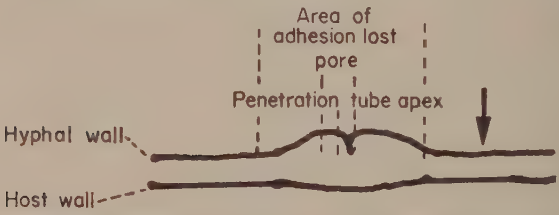


FIG. 2. Incomplete adhesion between fungus and host wall. (Original.)

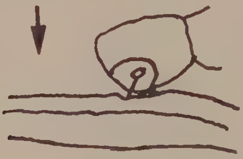
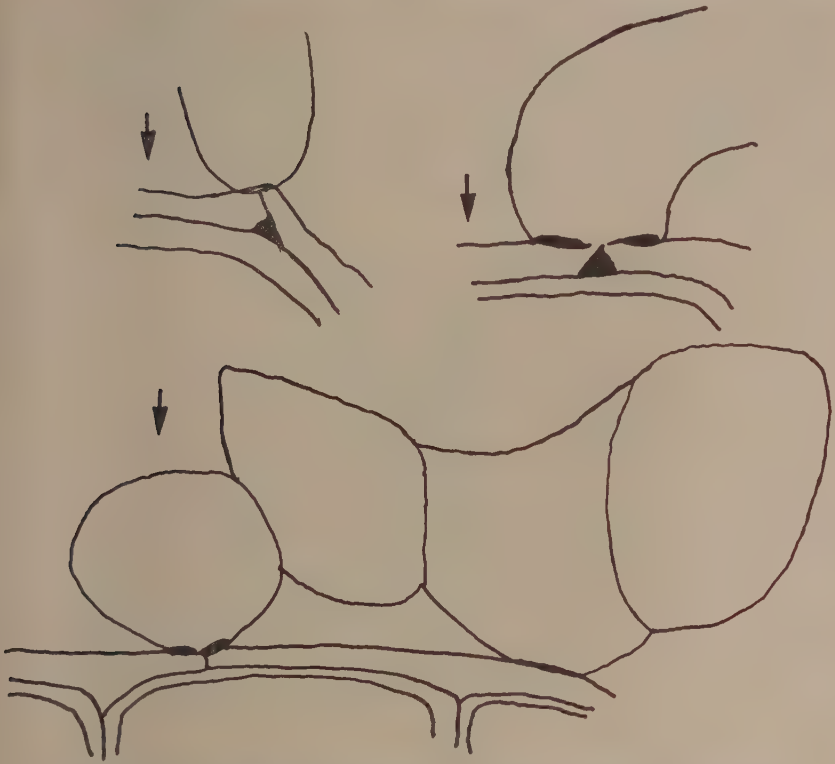


FIG. 3. Penetration failure by *Gymnosporangium juniperi-virginiana* into old apple leaf. (Magnification: × 850.) (After Nusbaum, 1935.)



FIGS. 4, 5, 6. Stages of penetration by *Venturia inaequalis*. (Magnification: × 1620.) (After Nusbaum and Keitt, 1938.)

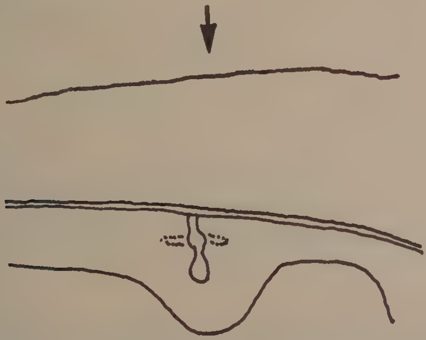


FIG. 7. Penetration tube passing through epidermal wall, *Erysiphe communis* on *Geranium maculatum*. (Magnification: × 1800.) (After Grant Smith, 1900.)

on a "thickened" ring round the pore. Their illustrations (see Figs. 4, 5, 6) show this area to cover nearly the whole of the adherent area. In both living *Puccinia triticina* and *Erysiphe graminis*, the writer observed a black ring surrounding the pore on artificial membranes. This black ring could be converted into a crescent form and back again to a ring by gentle movement of the lateral wall of the appressorium. It was deduced that in this area adhesion was lost, and the black ring was due to diffraction (Dickinson, 1949b). It seems to the writer that, particularly in view of its clearly lenticular form, Nusbaum and Keitt's thickened ring was really an area where adhesion had temporarily, or permanently, been lost. It must also be noted that frequently adhesion is lost in cytological preparations.

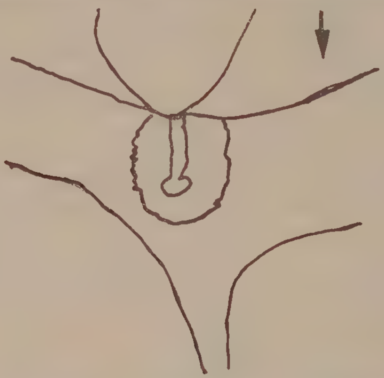


FIG. 8. Infection hypha of *Colletotrichum lindemuthianum* swollen into a vesicle in the disorganized cellulose layers. (Magnification: × 1500.) (After Dey, 1919.)

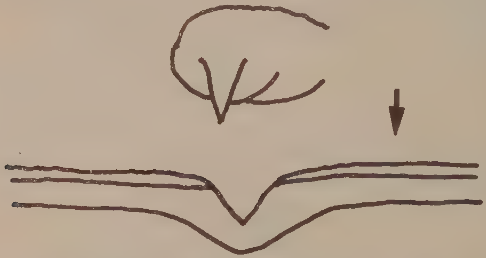


FIG. 9. Infection papilla of *Erysiphe graminis* in section with germ tube detached. (Magnification: × 2000.) (After Corner, 1935.)

The new growing point, it is to be presumed, is the only plastic area, while the rest of the appressorial wall will remain nonextendable. If penetration of the underlying host wall is easy, then no adhesion will be likely to be lost. But if penetration is difficult, the growing point will separate the appressorial and host walls. For an example where adhesion is not completely broken see Figs. 1 and 2. This is a less extreme case than Nusbaum's illustration (see Fig. 3). Under such conditions (see Fig. 2), with the adhesion beyond the ring round the pore still intact, indentation of the host wall could occur, just as well as indentation of the pathogen wall. The whole potential penetration would rest on the force due to the osmotic pressure of the fungus exercised on the very small diameter of the potential penetration tube. If such is the case, then there is no need to evoke intussusception or any other source of power for penetration. It should be emphasized that this hypothesis depends upon the assumptions that (a) only the very fine growing point is plastic, (b) adhesion is on occasion partly or wholly lost.

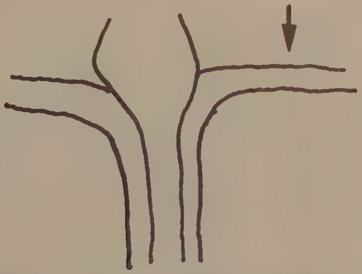


FIG. 10. Longitudinal section of wheat pericarp penetrated by *Ustilago tritici* with funnel at point of entry. (Magnification: × 1350.) (After Batts, 1955.)

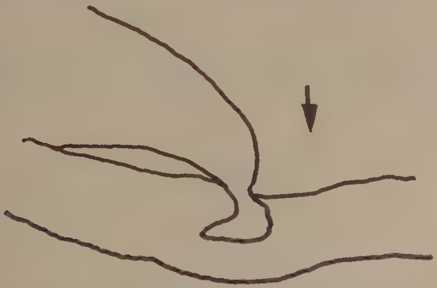


FIG. 11. Cuticle penetrated by a small peg of *Botrytis cinerea* spreading out in subcuticular layer. (Magnification: × 1200.) (After Blackman and Welsford, 1916.)

The diameter of the penetration tube during its passage through the cuticle has always been reported as very fine (see Figs. 4, 6). Drawings of penetration after the cuticle has been passed show that the penetration tube in the cuticle may increase in diameter (see Figs. 5, 9, 10). The diameter of the tube during its passage through the pectin-cellulose layers usually increases considerably in diameter (Figs. 7, 8, 11, 13) but does not always do so (Fig. 15). There is a clearly marked difference in outline between penetration in the smuts (Fig. 10), and that in *Botrytis cinerea* (Fig. 12). The former appears like an upright funnel, while the latter has the figure of an inverted funnel. Irregular variation in diameter has also been recorded (Fig. 14). An inverted funnel form was observed by Paddock (1953) in egress from the epidermal cell to the outside (Fig. 16). In this figure, Paddock included no cuticle, but his text and other figures indicate that it had been lifted off the epidermis.

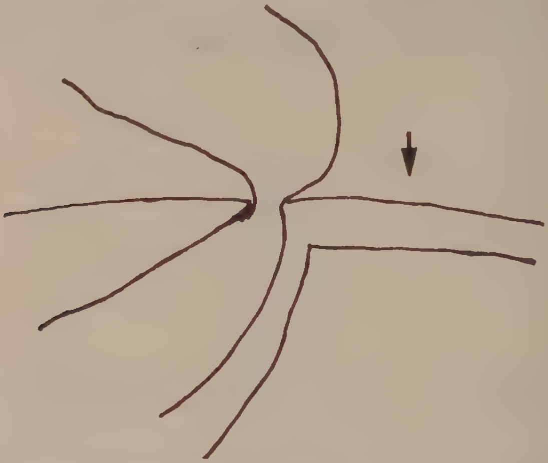


FIG. 12. Hypha of *Botrytis cinerea* growing in the subcuticular layer of the epidermal wall. (Magnification: × 1200.) (After Blackman and Welsford, 1916.)

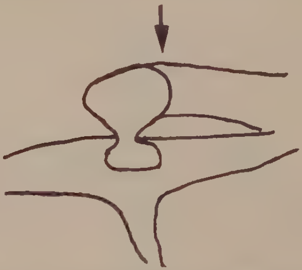


FIG. 13. Infection hypha of *Sclerotinia libertiana* has penetrated cuticle and is forming a vesicle. (Magnification: × 773.) (After Boyle, 1921.)

It is believed that these variations in the original diameter of the penetration tube in the cuticle, as well as the increase in diameter in the pectin-cellulose layers of the wall depend (a) on the ratio of pectin to cellulose, (b) on the potentialities of the enzyme system of the pathogen, and (c) on the potential force the pathogen can exert. When an upright funnel is produced (Fig. 10), it is suggested that the wall layers with a higher pectin-cellulose ratio are locally rendered very soft, while an inverted funnel form is produced by a less intense but more widespread softening and swelling.

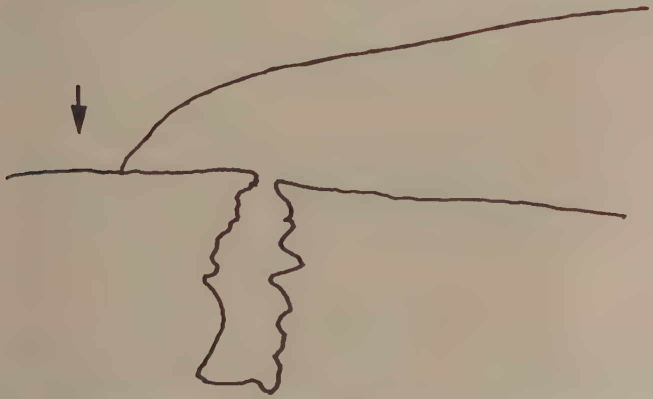


FIG. 14. Growth of infection hypha of *Venturia inaequalis* directly into the cuticle. (Magnification: × 1600.) (After Wiltshire, 1915.)

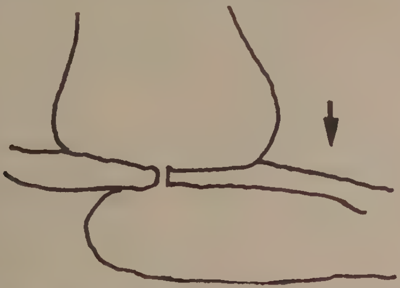


FIG. 15. Cell wall penetration by *Colletotrichium lindemuthianum*. (Magnification: × 1600.) (After Leach, 1923.)

Some observers have described penetration with no sign of appressorium. Penetration without adhesion could only happen with a really soft material. As long as force is required to penetrate the wall, there must be some adhesion. How large this adherent region has to be will depend on the efficiency of the adhesion, the force required to penetrate the underlying cell wall, and the diameter of the apex of the penetration tube. With a high angle of approach and an epidermis covered by a thin cuticle, it is quite conceivable that only a small area of adhesion is necessary, and further that the plasticity of the original growing point could be maintained. If, in addition, the pectin-cellulose part of the wall was readily softened before or after the cuticle had been penetrated and the cuticle itself was elastic, it is more than likely for an adhesive area to be formed around the original growing point without any halt in growth. The result of this would be a reduction of plasticity at the hyphal apex. As growth continued at the fine apex of the original growing point, the cuticle being readily penetrated and elastic, the original difference in diameter between parent hypha and penetration tube—which marked the adhesive region—would result in the disappearance of most signs of an appressorium or adhesive region (see Fig. 10).

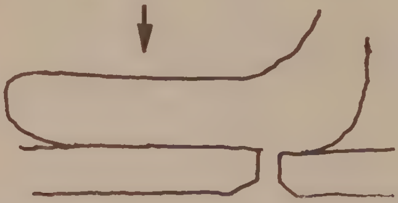


FIG. 16. Egress tube of *Helminthosporium victoriae* fanning out as it approached the outer part of the wall of an oat leaf hair. (Magnification: × 1500 approx.) (After Paddock, 1953.)

In nematodes, as in the fungi, to achieve penetration by force, it is essential that there be some method of compensating for the equal and opposite back thrust to the applied force. This can be achieved by anchorage or adhesion. In nematodes attacking root tips, the hypothesis generally accepted has been that put forward by Linford (1942) in describing entry of the root rot nematode. He wrote "a larva thrusts its stylet chiefly when its body is so braced that it may hold its head firmly against a resistant surface." He made no mention of how such bracing was to be achieved. Since then, suggestions have been made that either the surface tension of the water film or the inertia of the soil particles adjacent to the larva might provide the required bracing. In an attempt (Dickinson, 1959) to find out how nematode larvae penetrate the outer cell wall of a root, drops of larval suspensions (*Heterodera schachtii*) were placed on cell wall imitations, e.g., membranes made of nitrocellulose, etc. Three stages in penetration were observed, adhesion by suction, assumption of a vertical position, and finally penetration by stylet extrusion. Following contact with a membrane, and in a reasonable depth of liquid, larvae moved freely along the surface and made attempts at adhesion by pushing the fused lips against the membrane. Contact between the fused lips and a hydrophobic surface led to adhesion by suction. After successful adhesion, the body was hoisted to a position vertical to the membrane. While the head remained stationary and fixed to the membrane, the rear part of the body swayed or rotated slowly round and round. This is reminiscent of the method of penetration by *Schistosoma mansoni,* a member of the allied group, Cestoda (Gordon and Griffiths, 1951). Stylet extrusion followed, and the membrane was seen to have been penetrated. Finally, as Linford (1937) observed, saliva was ejected. This was seen below the membrane. Linford (1942) described the passage of cell walls as being achieved by a combination of stylet penetration and cell wall dissolution by the saliva. Passage through membranes has been seen, but the actual effect of the saliva has not yet been determined.

III. THE BREACHING OF THE BARRIERS

In reviewing the available data on the breaching of barriers, a distinction can obviously be made between those barriers which appear to be wholly or partly breached by force and those which are not breached by force. When an external barrier is breached, there is clearly a difference in the conditions outside and inside it. Such a difference does not necessarily apply to internal barriers. With external barriers, their permeability and solubility are of major significance. In internal barriers, the passive or active behavior of the host is of major importance. Barriers in the breaching of which force is used are few in number but with further research are likely to increase considerably.

For the purposes of this text, barriers have been classified as follows:

A. Barriers Breached Wholly, or in Part, by Force
  1. External Barriers
    a. Barrier Impermeable and Insoluble
    b. Barrier Impermeable but Soluble
    c. Barrier Apparently Permeable and Insoluble
    d. Barrier Apparently Permeable and Partly Soluble
    e. Barrier Due to Tensile Strength
    f. Barrier Having a Hydrophilic Surface
  2. Internal Barriers
    a. Preinfection Barrier
    b. Postinfection Barrier
      (1) Host active
      (2) Host passive

B. Barriers Not Breached by Force
  1. Morphological Barrier
  2. Barrier Due to the Presence of a Hydrophobic Surface
  3. Barrier Due to Absorption of a Pathogen's Enzyme System

A. *Barriers Breached Wholly, or in Part, by Force*

1. *External Barriers*

a. *Barrier Impermeable and Insoluble.* The breaching of an impermeable and insoluble barrier should cause no reaction, as determined by cytological or chemical tests, other than that of actual passage following adhesion. The width of the outer epidermal wall should show no alteration in the neighborhood of the penetration. The diameter of the penetration tube should also not change from that of the actual apex of the tube. An alteration in diameter of the penetration tube on passing from the cutin to the pectin-cellulose "layers" of the wall would usually be considered as evidence of enzyme action. But it is not impossible that the tensile strength, resistance to deformation, etc. of the two "layers" might be so different as to cause a change in diameter. If this were so, it would be expected to be accompanied by negative staining reactions in the cell wall. In general, it seems best to adopt a conservative view. While realizing the many possible examples in the literature, I suggest that the criteria required are only fulfilled in the passage of the sporidial penetration tubes of *Puccinia graminis* through the outer epidermal wall of the leaves of *Berberis vulgaris* (Waterhouse, 1921). Waterhouse observed no sign of any change in the diameter of the penetration tube. He could detect no staining reaction in either the cuticle or the pectin-cellulose part of the wall.

b. *Barrier Impermeable but Soluble.* When breaching is accompanied by complete dissolution of the barrier, it would appear at first that force as such, was not concerned in the process. In both *Phymatotrichum omnivorum* (Watkins, 1938a) and *Endothia parasitica* (Bramble, 1936) there is no evidence of effect on the cells within the barrier. The penetration is achieved by tangential growth through the barrier. Following the by-passing of a layer of suberized tissue, this loses its coherence and disintegrates. Such evidence suggests a cellulytic enzyme system. A most noteworthy feature of *P. omnivorum* is its wide host range, suggesting a mechanism of penetration capable of considerable variation together with catholic metabolic requirements. Comparison between the enzyme systems of it and *E. parasitica* should yield interesting results.

c. *Barrier Apparently Permeable and Insoluble.* The first example so far found is that drawn by Grant Smith (1900) of *Erysiphe communis* after adhesion to an epidermal cell of *Geranium maculatum*. This and his description suggest that he observed a stain reaction in the cell wall before he was able to see any penetration tube having passed through the cuticle. Such an observation must be treated with caution, but not only on account of its negative nature. Other evidence was provided by Chaudhuri (1935) who claimed that—by staining methods—he had demonstrated the passage of some substance through the unperforated cuticle, which affected the underlying cell wall. Paddock (1953), more recently, has shown clearly that with a strain of *Helminthosporium victoriae* on the oat variety Victoria the protoplasm of the epidermal cell was visibly affected some 12–18 hours before the pathogen passed through the cuticle and entered the cell. Finally, Kerr and Flentje (1957) have demonstrated that the penetration tubes of *Pellicularia filamentosa* were not formed unless there was a passage of some substance from within the epidermis to the outside. They considered that there was an essential physical as well as chemical stimulus to penetration. Brown (1922) showed clearly that when water drops were put on the outside of petals of various species, their conductivity was raised by the passage of electrolytes through the cuticle. Further, he showed that the germination of *Botrytis cinerea* might be raised or lowered in such water drops. Later work has suggested that passage through the cuticle either way is fairly strictly limited. While, by chance, larger molecules may pass through a crack, normally, passage would be confined to crystalloids.

With direct observation or by the use of stains these effects might be deduced to be due to pressure, after adhesion, on the host cell wall, instead of the passage of substances through the cuticle and outer epidermal wall. But Brown (1922) and Kerr and Flentje (1957) have both demonstrated the effect experimentally and consequently it would appear much more likely that passage of substances affecting the cell wall or the protoplast occurs rather than a reaction to pressure.

d. *Barrier Apparently Permeable and Partly Soluble.* Thomas (1934), in his description of the entry of the rhizomorph of *Armillaria mellea* into the root of walnut, has remarked on an effect on the cells below the suberized cell layers before anything more than adhesion between host and pathogen had occurred. As previously, here again there is the possibility that such an effect could be achieved by pressure after adhesion, although Thomas's description does not suggest this interpretation. There is also the likely, but as yet undemonstrated, existence of crevices in the suberized layers—perhaps due to internal tissue strains—through which passage of substances capable of affecting the underlying host tissue could pass. The subsequent dissolution of the suberized layer was described by Thomas as a gradual mass "sinking in" and passage through the suberized layer. Subsequent to passage through the suberized layer, individual hyphae grow out into the surrounding parenchyma. It is not known whether in fact *A. mellea* possesses a sufficiently strong cellulytic system to at least soften or disrupt the suberized cell walls, and allow the amorphous suberin material itself to be pushed aside. This example is in contrast to the penetration by *Phymatotrichum omnivorum* and *Endothia parasitica* previously described, where tangential growth was the means of passage through the barrier, followed later by disintegration.

Before Brown's work in 1915, careful examination had always been made for evidence of passage of substances toxic to the plant. Since the publication of Brown's paper, it has been generally accepted that there is no passage of substances through the cuticle other than water of transpiration. In fact the evidence suggests the passage of crystalloids up to a certain molecular volume in either direction. The evidence for partial dissolution or softening of the barrier is usually that such an effect has taken place after the cuticle had been perforated. It is to be hoped that in the future, such evidence will be examined critically. But most observers (Blackman and Wellsford, 1916; Wiltshire, 1915, and others) do report an indentation of the outer epidermal wall. It is not known whether, following adhesion, there is passage of some substance which affects, to a small degree, the strength of the wall, thus allowing the indenting of the epidermal wall. These authors also cite frequent entry at a point above the anticlinal walls.

Hansford (1946), in his examination of the foliicolous Ascomycetes, said of the family Chaetothyriaceae: "In some species sections cut transversely through the leaf show that the cuticle beneath some hyphae is somewhat swollen, and it is thus possible that some species at least absorb nourishment from the leaf by chemical action upon the host cuticle to render it permeable." Again, in his description of *Microcallis nuxiae* (Doidge), Hansford has written "there is no penetration of the stomata, but in places the hyphae are very clearly attached to the cuticle, which appears to be partly dissolved, when detached from the leaf the mycelium leaves the lower walls of many cells adherent to the cuticle." Hansford clearly envisages some erosion of the cuticle, even though in *Microcallis*, his evidence might be just the result of a high degree of adhesion.

e. *Barrier Due to Tensile Strength.* Hawkins and Harvey (1919) were the first to test the tensile strength of barriers by means of a fine glass rod held at the end of a Joly spring balance. They measured the force required to penetrate potato tissue, both resistant and susceptible to *Pythium debaryanum*. The measurements were of the weight per unit area required to penetrate. In addition to demonstrating that resistant tubers required a greater weight per unit area for penetration than susceptible tubers, they also showed that there was no significant difference in cell size, but that *P. debaryanum* grew more quickly through susceptible tissue. They determined that the crude fiber content of resistant tubers was higher than in susceptible tubers. They ascribed the resistance in the tuber to be due to the fact that the osmotic pressure in the fungus was lower than that required to penetrate resistant tubers, but higher than that required for penetration of susceptible tubers. Supposing all their measurements and conclusions are correct, it has to be concluded that some softening of the cell walls took place, through enzyme action. Similar studies, but not in such detail, have been made by Rosenbaum and Sando (1920) using *Macrosporium* spp. on tomato fruits, and by Melander and Craigie (1927) with *Puccinia graminis* on various species of *Berberis*. Yoshi (1941) studied the resistance of rice to blast disease caused by *Piricularia oryzae*. He was able to show that resistance was proportional to the amount of nitrogen in the leaf. However, he could find little or no correlation between resistance to *P. oryzeae* and toughness, as measured by force required to penetrate, of any given area of leaf. It would seem quite possible in view of the silica content that if he had measured the force required to deform, he would have found the correlation for which he was searching.

It is probable that examples of entry limited to special organs would, if thoroughly investigated, be found to be due to the immaturity and consequent lack of tensile strength of the organ tissues involved. For example, in nature, *Claviceps purpurea* is limited to ovaries, *Plasmodiophora brassicae* to young roots or root hairs, *Heterodera schachtii* to young root apices, etc. Experimentally, there is found to be no such limitation, although infection of all types of organs has not yet been demonstrated.

A rather different example is that demonstrated by Newton and Brown (1934). They showed that the specific range of some rust species could be widely extended by injecting their uredospores into young growing points. This is undoubtedly an example of immature tissues being more susceptible, but it is unlikely that it is due solely to the low tensile strength of the tissues concerned.

f. *Barrier Having a Hydrophilic Surface.* Root hairs have so far never been observed to be entered by the larvae of nematodes. Their walls are said to be made up of calcium pectate and cellulose (Cormack, 1949), which would result in their surfaces being hydrophilic. As larvae of *Heterodera schachtii* have been shown to be unable to adhere successfully to a hydrophilic surface, this might also apply to root hair surfaces for the same reason. There is, however, the possibility that the ratio of the diameters of the fused lips and root hairs might be too large for adhesion, that is that the convexity of the root hairs might be too great for the fused lips to complete an adhesion ring. This would prevent suction being exerted. In either case, the suggested failure to enter root hairs by force would be due to a physical cause.

2. *Internal Barriers*

Once entry is achieved, there are still some preinfection barriers, which may either prevent or limit the spread of the pathogen by reason of their impermeability, insolubility, or impenetrability. Postinfection barriers can prevent or limit the spread of the pathogen by reason of their impermeability, etc., but are more easily classified according to the host reaction. The two classes consist of (a) those in which there is active host reaction, new tissues being formed, (b) those in which there is passive host reaction not involving an increase in number of cells.

a. *Preinfection Barrier.* The endodermis is an impervious barrier existing in the host previous to infection. Pearson (1931), in describing the progress of *Gibberella saubinetii (G. zeae),* and Simmonds (1928), using *Fusarium culmorum,* observed the passage of the pathogen through the cortex to the endodermis. In front of this layer of cells not only was there an increase in number of hyphae before the barrier was passed, but the parenchyma walls were more swollen. There were many fewer hyphae in the middle of the cortex, and the host walls were less swollen. Finally, the hyphae passed through the endodermis via the passage cells, but in the meantime this had apparently acted as an impervious barrier, with a resultant increase in pectolytic enzyme concentration and presumably, in consequence, an increase in wall swelling.

Another type of a preinfection barrier is that described by Hart (1931). In the stems of some varieties of wheat, the chlorenchyma is enclosed by a longitudinal sclerenchymatous sheath except toward the epidermis. The black stem rust pathogen does not penetrate this barrier and so is confined to the strips of chlorenchyma running up and down the stem. The failure of the pathogen to pass through such bands of sclerenchyma may well be due to the cellulose-lignin content of the cell walls which makes their tensile strength too high for penetration without some preliminary softening process. This the pathogen cannot do as it lacks the necessary cellulytic-lignolytic enzyme system.

b. *Postinfection Barrier.* In both (a) and (b), the start of the host reaction is the same, *viz.*, the swelling (sometimes very slight) and, in addition, the softening of the host wall in the neighborhood of the pathogen. In both, the success or failure of the pathogen to breach the barrier depends on the relative rate of its growth as compared to that of the barrier's formation. Sometimes a complication is present in that, for a period, nutritional or chemical factors may reduce the rate of growth of the pathogen relative to that of the barrier's formation.

(i) *Host reaction resulting in cell division.* Both Cunningham (1928) and Wardlaw (1930) have described such host reactions in considerable detail, the former in leaf tissues, the latter in the cortex and vascular cylinder of banana rhizomes. In all cases, the essential final result is the formation of an impervious and impenetrable barrier to the further progress of the pathogen. The successive features of the host reaction in parenchyma tissues are, first, the swelling of the walls. These cells then increase in volume and fill up the intercellular spaces. The walls of the cells adjacent to the swollen cells but further away from the pathogen become slightly suberized. Beyond them, the cells begin to divide forming a cambiform layer the walls of which become suberized and form a cork layer at right angles to the path of the enzyme system diffusing from the pathogen. The original swellings of the walls were probably the result of a pectolytic enzyme system; coincident with this, there would be a softening of the walls. As a result, the wall pressure : osmotic pressure balance is altered and this accounts for the increase in the volume of the cells and consequent decrease in intercellular space. During this period, many of the fungi used by Cunningham were intercellular and necrosis was considerably delayed. When, however, the hyphae are intracellular, necrosis sets in early accompanied in many cases, as for example, *Fusarium oxysporum f. cubense*, by a blue-black color. The pathogen grows up to the suberized layer, and if this is complete before its arrival, then it is unable to breach the barrier. If, however, as is usually the case in zonate cankers, the barrier is incomplete, then the pathogen grows around the impervious parts, and continues its advance with a similar series of reactions in the newly invaded tissues. In pathogens which have a cellulytic enzyme system, the successive barriers are more readily breached.

In the above circumstances, it will be readily appreciated how important are the relative rates of growth of the pathogen and formation of the barrier. Naturally, such a balance is readily tilted one way or the other by the effect of relatively slight environmental changes on the host-pathogen complex, as well as in the particular characteristics of the host or pathogen.

The means of preventing entry through vascular tissues has been clearly described by Wardlaw (1930). The essential features are the formation of tyloses in the vessels and their subsequent lignification, prior to the arrival of the pathogen. Vessels may also be blocked by their collapse owing to the increase in volume of the neighboring xylem parenchyma followed again by lignification. This new barrier is impenetrable, and is probably impermeable to the pathogen's enzyme system. Breaching such a barrier again, only takes place by a change in the relative growth rate of pathogen and barrier formation, which are, of course, considerably dependent on environmental conditions.

(ii) *Host reactions not resulting in cell division.* Here, where the host reaction is limited to some form of wall swelling, the prospects of successful barrier formation are much less satisfactory. The range and variety of wall swelling, principally in parenchyma tissues, is very great. In considering such swelling, the structure of the cell wall with its middle lamella and rising ratio of cellulose to pectin toward the lumen, must always be in mind.

In Allen's description (1927) of the attack of *Puccinia triticina* on Malakoff wheat she observed swelling of what appeared to be blocks of middle lamella (pectate) in the adjacent cell walls of the mesophyll at a considerable distance from the infection site. In contrast, in citrus melanose Bach and Wolf (1928) described the swelling to a gummy substance of the primary walls, with almost the occlusion of the lumen. In *Venturia inaequalis*, Marsh and Walker (1932) have recorded that in the stem, the lumen of the epidermis may be almost completely occluded. The solution of the middle lamella, and consequent lack of coherence between the epidermis and pallisade cells of the leaf, is a characteristic feature of silver leaf of fruit trees (Tetley, 1932). This observation is remarkable for it illustrates the distance which the enzyme travels and its persistence in the stem.

The swelling may be more localized, as Batts (1955) has shown in his recent study of loose smut. Such hyphal sheathing by host wall material has long been known, but hitherto it has been thought of as a deposition of fresh wall material. The callosities (Young, 1926), now usually called lignitubers, formed around invading hyphae should be mentioned, as well as the observation (Fellows, 1928) that, locally, the middle lamella is very swollen. Here, failure to breach the barrier has been recorded by Fellows (1928) and by Young (1926). The essential feature is the similarity to a local reaction as in the hyphal sheathing in smut pathogens, the difference being that as a rule the lignituber does not invaginate far into the cell.

Such swellings in parenchyma cell walls are probably due to pectolytic enzyme production by the pathogen—with, as a rule, its movement between the cells and not into the cells. Such a path combined with the rising ratio of cellulose to pectin toward the lumen enables most histological preparations to be understood. Paddock's description (1953) of the egress of the sporulating hyphae of *Helminthosporium victoriae* from the leaf makes it clear that the innermost cellulose layer was the least easily penetrated.

Before considering such swellings as possible barriers, the question of their origin must be discussed. Does the original cell wall swell and consequently soften or, as many have suggested, is there new cell material deposited around or in advance of the pathogen's penetration?

A clear example of new wall layer deposition is in Moss's (1926) description: first the maturation of the haustoria in *Pucciniastrum* spp., and then subsequent to maturation, the formation of host wall material around the mature haustoria, with the presumably consequent degradation of the haustoria. Most other examples of swelling, even those in powdery mildews (Smith, 1938), can be more readily interpreted as being due to swelling of either the pectate layer or of the cellulose-pectin substance layers in the cell wall. But the innermost cellulose layer cannot readily be penetrated without a cellulytic enzyme system, and as it is elastic, it is pushed in advance of the hyphal tip just as a finger may invaginate a rubber balloon. If with such an interpretation in mind, Batts' (1955) specimens are examined, it will be seen that at the point where the appressorium for the passage of an internal wall is formed, there is a line which is the line of junction between the cellulose of the preceding penetrated wall, and that of the cell wall material which will become swollen.

Undoubtedly, Young's (1926) description of lignitubers suggested that in inappropriate host-pathogen complexes, such barriers could be successful. Further, Fellows' (1928) description also suggested that such might sometimes be the case of *Ophiobolus graminis* in wheat. In general, it must be admitted that the process is less of a defensive mechanism and more of a local host reaction. Its relative frequency combined with the fact that there is undoubtedly an inextricable mixture of force and enzyme action, has made it essential for it to be dealt with in some detail in this chapter.

For the parallel phenomena in woody tissues, the work of Brooks and Brenchley (1931) may be cited. For infection by *Stereum purpureum* they showed that gummy material formed as a result of enzyme activity on vascular elements was an effective barrier. Similarly, Willison

(1931) observed gum material formed from preexisting cell walls to be penetrated by *Cytospora* sp. by means of appressoria and penetration tubes.

B. *Barriers Not Breached by Force*

There is no external barrier when normal entry is through natural openings or wounds. The hair covering of leaves would appear to be a self-evident protectant, but no proven examples have been recorded. The architecture of the vessels, a morphological feature, has been correlated with some delay in the spread of a pathogen through the host. The waxy hydrophobic covering of leaves has been considered as a potential method of disease escape. The barrier achieved by absorption of the pathogen's enzymes still requires more evidence before it can be fully accepted, just as the possible effect of the macromolecular configuration of the cell surface is at present a subject of investigation.

1. *Morphological Barrier*

The presence of hairs on the surface of a resistant leaf as compared with a hairless surface of a susceptible leaf, suggests itself as a self-evident example of a morphological barrier. The barrier's effect is either to ensure that the pathogen exhausts its food reserve before reaching a surface through which it can enter the leaf, or induces a microclimate which may be unsuitable for the pathogen. No evidence of such cases has as yet been found proven in the literature. Johnstone (1931) considered such a feature to be a possible resistance mechanism or barrier to an attack of apple scab.

A rough, as opposed to a smooth, surface might be considered to have some effect in as much as many fungi entering through the epidermis seem to penetrate opposite the anticlinal walls of the epidermis, as opposed to the central part of the outer epidermal wall of the cell. Such a feature might also influence the entry of an eelworm larva, in as much as entry is often reported as being opposite the anticlinal walls, that is in the angle between two of the cells of the outer layer toward the root aspices.

More convincing evidence was found in examples where the architecture, particularly of the vessels, influences the spread of the pathogen in the vascular system. White oaks are more resistant than red oaks to oak wilt caused by *Chalara quercina* (Young, 1949). This, it is suggested, is because the diameter of the vessels is smaller and occlusion by tyloses more frequent in the former than in the latter. Varieties of sugar cane, resistant to the attack of *Physalospora tucumanensis* (Edgerton, 1950) have the septations in the vessels intact and consequently the rapid spread by conidia is prevented. The mycelial spread is centrifugal from the points where the conidia lodge. In varieties of lucerne, e.g., Grimm, which are resistant to alfalfa wilt, caused by *Corynebacterium insidiosum* (Peltier and Schroeder, 1932), the septations of the vessels are incompletely dissolved away, and the xylem is more compact, with fewer intercellular spaces than that of the susceptible varieties.

2. *Barrier Due to the Presence of a Hydrophobic Surface*

The presence of wax on the surface of a leaf has been suggested as a resistance mechanism in plants. Such a surface, or for that matter a highly polished cuticle, makes the formation of a water film or the retention of a water drop, much more difficult than on a nonwaxy or unpolished cuticle. Such a difference exists between pea and bean leaves, water films forming much more readily on the latter. Certainly, such a condition would reduce the opportunity for entry by pathogens requiring a water film.

Such surfaces may produce their effect by rendering exomosis into a water film less likely. Brown (1922), measuring the conductivity of water drops on leaves and petals, pointed out that there was always an air film between water drops and some petals like *Rosa* and *Viola.* It is probable that this had an actual effect on exosmosis, but whether such was sufficient to prevent attack by *Botrytis cinerea* was not shown.

Wingard (1941) grew an unnamed barley variety on soils ranging from normal garden soil to one with a 2% content of alkaline salts. He found that less rust infection took place on the plants grown in the alkaline soils, but that where infection did take place, it was very vigorous. He used a spray of spore suspensions for innoculation, and attributed the results to the greater bloom, due to wax, on the plants grown on the alkaline soil. This he suggested allowed a much greater run-off of water droplets and spores, than on the plants grown on garden soils.

The hydrophobic surface which lines the intercellular spaces in plants provides much more convincing evidence that a hydrophobic surface does confer resistance. The presence of such a surface was clearly demonstrated by Lewis (1938), although earlier both Priestly (1943) and Ursprung (1925) showed the presence of cutin in such cell walls, and Scott (1950) has suggested the presence of a layer of suberin lining the intercellular spaces. This hydrophobic surface of the intercellular spaces would affect the rate of spread of bacteria in them. This is supported by the work of Shaw (1934) on apple fire blight, by Clayton (1936) and later Johnson *et al.* (1940) on black fire of tobacco. All these authors showed that the internal humidity, not merely the water logging or water congestion of the leaves, was a major factor in determining the rapid spread of the pathogens. Under high humidity conditions, perhaps together with a fall in temperature, the hydrophobic surface on occasion was unable to prevent a water film being formed. When such conditions occurred, after entry of bacteria through stomata or wounds, they spread rapidly through the tissues. By artificial water congestion, Johnson (1937) enabled *Bacterium angulatum*, to which tobacco was normally resistant, to spread rapidly through the leaf. In addition, other bacteria, even *Escherichia coli*, were able to attack a wide range of host leaves causing necrosis.

The reason why such attacks do not normally take place is that the high angle of contact between a water drop and a hydrophobic surface prevents the spread of the drops over the surface. Even though bacteria of many kinds may be found in storage tissues, spread is very slow or absent unless, or until, this hydrophobic surface effect is overcome.

3. *Barrier Due to Absorption of a Pathogen's Enzyme System*

In 1915, Brown described the effect of placing a small amount of a suspension of enzyme extracts from *Botrytis cinerea* germ tubes on washed discs of freshly cut potato tubers. The volume of enzyme suspension was small relative to the area of the surface of the disc. The relative humidity surrounding the discs was kept as high as possible. Under such conditions, no rot developed. If, however, the discs were injected with water, complete rotting, i.e., lack of cohesion between the cells, took place. Later necrosis set in. Brown (1936) considered the freshly washed discs to be subturgid, and the injected ones turgid. He attributed the failure of rotting to develop on the subturgid tissue as being due to some type of inactivation of the enzyme system. The results of using *Bacterium carotovorum* and *Pythium debaryanum* and their enzyme extracts were different only in that the degree of attack on the freshly washed discs was not entirely negative.

Gregg (1952) later showed that soaking the tuber tissue under water for 6 hours raised the water content almost to that of the injected tissue, but that the amount of rotting was much less. These results have been further investigated by Fernando and Stevenson, (1952), Gregg (1952) and others, while a general description of them was included in an address by Brown (1955).

Only exceptionally are potato tubers in a fully turgid condition before cutting and washing, etc. The potential water uptake may be as much as 8% fresh weight or more while the intercellular spaces only occupy 1% by volume of the potato tuber. Part of this is very rapidly taken up as soon as the freshly cut surface is immersed in water and the half time of weight increase may well be as low as 30 minutes or more. The washing of the potato discs, or tissue, for 15 minutes was done to remove broken or injured cells (Brown 1915). Assuming this to have been done efficiently, then an enzyme drop on the surface of such a tissue would be in contact almost entirely with the cellulose inner wall, and would be in contact with only a small amount of middle lamella pectate, and pectic substance in the actual cut cell walls. It should be remembered that an enzyme suspension cannot pass across a cell wall, but only along the pectate and pectin-cellulose parts of the wall after reacting upon it. The innermost cellulose wall layer excepting the plasmadesmata has always been believed to be impervious to all but crystalloids.

Regarding the difference between the rotting in injected as compared with soaked tissues, there was a difference of 1% or so in the water content, and this may well have been due to only the former having the intercellular spaces filled with water. The difference in rotting was about the same as that between soaked and washed tissues.

No doubt the start of suberization might well be delayed by the low O_2 tension in the injected tissues. But as the intercellular spaces of the soaked tissues were filled with air, the hydrophobic surface of these intercellular spaces would prevent the rapid spread of the suspension through these tissues, in contrast to that in the injected tissues. The difference in amount of rotting between injected and soaked tissues could be due to a combination of low O_2 tension and easy spread of the suspension in the injected tissues.

The other comparison to be made is that between tissues soaked under water for 6 hours and those washed for about 15 minutes. Between these two tissue conditions there was a large difference in water content as well as in amount of rotting. The effect of a small amount of enzyme suspension on both tissues would be that action on the pectate and pectin substance part would start, and such action would readily continue in the soaked tissues. In the lower water content tissues, there would be a greater demand by the suction pressure of the cells than the enzyme suspension could be expected to withstand—not having any osmotic pressure. Consequently, the enzyme suspension would soon become concentrated and dried up. In other experiments it was found that rotting would develop on freshly washed discs if sterile water was added periodically to counteract the drying up of the suspension drop which took place in spite of the relative humidity per cent being as high as possible. Addition of glucose or sucrose also prevented drying up, and so allowed some rot to develop. The enzyme suspension was apparently not completely inactivated until about 8 hours or more after the suspension had been put in place.

The difference in water content or water absorptive capacity could, therefore, be the reason for the lack of rotting in the freshly washed as compared with the rotting in the soaked tissues.

If this suggested absorption barrier is a correct interpretation of the detailed experiments by Brown (1915), Fernando and Stevenson (1952), Gregg (1952), etc., then it is not impossible that such a barrier might be acting in other host-pathogen complexes (Brown, 1955). Any likely complex would necessitate that rotting or lack of cohesion between the cells was a major symptom, together with an almost wholly intercellular pathogen and as direct a relation as possible with the water content of the tissues or with the relative humidity around the host tissues. A possible example may be susceptible tomato plants attacked by *Cladosporium fulvum*, which are readily infected between 90% relative humidity or over, and not easily infected at 75 to 80% relative humidity (Small, 1928). Bond (1936) has shown that some strains of the pathogen are almost entirely intercellular and that only slight, if any, necrosis develops at sporulation. Another intercellular pathogen, the parasitism of which has been suggested as being affected by water content, is *Venturia inaequalis*. Marsh and Walker (1932), when comparing the relative depth of penetration of the leaf and stem tissues, suggested that the greater depth of penetration in stems might be due to their higher water content.

IV. Conclusion

The breaching of the host's barriers by physical means is one of the ways in which the disease resistance mechanism of a host is overcome. A disease resistance mechanism may be simple and physical in nature, as shown by Melander and Craigie (1927) in their study of the tensile strength of the outer walls of barberry species, or it may be simple and chemical in nature as in Walker's (1923) demonstration of the effect on *Colletotrichum circinans* of protocatechuic acid, extracted from colored scales of onions. Other disease resistance mechanisms are more complex. It is likely that some of the complex disease resistance mechanisms may consist of a chain of reactions, part physical and part chemical in nature. These conclusions immediately raise the question whether the physical or static characteristics of the host, as compared with the chemical or dynamic ones, are altered less by environment and consequently of greater value in breeding for disease resistance.

In this chapter there has been much speculation. This approach has been deliberate. It is hoped that it will not lead to controversy on paper but will stimulate further experimental work to test the validity of the suggested hypotheses.

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CHAPTER 7

Chemical Ability to Breach the Host Barriers

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I. INTRODUCTION

The living plant growing vigorously is an uncongenial substrate for the growth of most microorganisms. The properties which make it uncongenial can be regarded as barriers to infection and colonization, and the total of these properties is resistance to disease. If a microorganism invades a plant and continues to grow in the tissues, it must be assumed either that it is unaffected by the barriers which exist for other microorganisms, or that it possesses mechanisms which make these barriers ineffective. Some barriers can be recognized with a reasonable degree of certainty, and where this is so, it is generally not difficult to show whether or not a particular microorganism possesses the ability to overcome them, and does so under natural conditions. Thus, it is reasonable to suppose that cell walls form barriers to the movement of bacteria through plant tissues so that an ability to degrade essential components of cell walls will be a factor determining pathogenicity. More often, however, the properties of tissues which prevent establishment and growth of an organism are more subtle in character, and only in a few instances have they been recognized. It follows that correspondingly little is known about the properties which enable some microorganisms to produce diseases in such tissues.

Clearly, the subject of this paper encompasses much of what is known about the physiology of infection of plants by fungi and bacteria. Because of this it will be dealt with in general terms.

II. ACTIVITY OF THE PATHOGEN AT THE SURFACE OF THE PLANT

A. *Effect of Substances Secreted by the Host*

Before invasion, a pathogen at the surface of a plant is in an environment which may be modified substantially by the plant itself. The influence of plant roots in this way was recognized by Hiltner (1904) who introduced the term rhizosphere for the part of the soil modified by the roots. Many people have since shown that secretions from roots, or sloughed-off parts of roots, can alter, quantatively and qualitatively, the flora at and near the root surface. Such effects depend on the plant species, age of the plant, and the conditions in which it is growing, and it is not difficult to envisage how these effects could alter the activity of a pathogen. Thus, the flora of the rhizosphere may be changed to a type in which the pathogen is less likely to grow and multiply, as described for varieties of flax and tobacco having different susceptibilities to *Fusarium oxysporum* f. *lini*, and *Thielaviopsis basicola*, respectively (Timonin, 1940; Lochhead *et al.*, 1940). The rhizospheres of resistant varieties contain a greater number of organisms, and these organisms are different from those in the rhizospheres of susceptible varieties. Timonin (1941) also showed that solutions in which roots of a resistant variety of flax had grown contained 25 to 37 mg. HCN per plant grown, and supported relatively good growth of *Trichoderma* *viride*, but not of *Fusarium culmorum* and *Helminthosporium sativum;* susceptible plants produced only a trace of HCN. *T. viride* is antagonistic to many fungi in acid soils, and resistance was in part attributed to the enhanced activity of this fungus in the rhizosphere.

Similar results have been obtained with a number of other host-pathogen combinations and in each it is possible that the host, by stimulating growth of one or more antagonists, creates a barrier to infection at the root surface. The ability of a pathogen to overcome this barrier rests in its capacity to grow in the face of this antagonism. When antagonism depends on the secretion of an antibiotic, something often suggested but not yet proven conclusively, organisms not affected by this barrier are those insensitive to the antibiotic produced.

Root secretions may also affect pathogens more directly. This was shown by Buxton (1957) with varieties of peas differing in their susceptibilities to different races of *Fusarium oxysporum* f. *pisi*. Exudates from the roots of one variety reduce the germination of spores of a race only weakly pathogenic to the variety, but have far less effect on the spores of virulent races. The substances secreted do not greatly affect hyphal growth and do not prevent germination entirely; their significance as barriers to infection, therefore, remains to be assessed. Clearly, however, they and similar substances, could be important when spore germination is reduced for other reasons; under such conditions they could prevent infection by inhibiting germination.

At the intact surface of the shoot the variety and amount of materials of host origin is probably far less than on surfaces of roots. This is because the cuticle is relatively impermeable, because materials corresponding to the dead outer tissues of roots are absent, and because deposits are periodically removed by rain. There is now no doubt that shoot surfaces carry a substantial flora (Last, 1955). This flora is not likely to be as active or as varied as that on roots but under the suboptimal conditions for growth at the surfaces of shoots, secretions by the saprophytic flora might reduce substantially the growth of the pathogen.

Growth of pathogens may also be reduced or prevented by substances of host origin present at the surface of leaves. Hafiz (1952) showed that certain varieties of *Cicer arietinum* are resistant to attack by *Mycosphaerella rabei* because they carry more glandular hairs per unit area, and because these hairs secreted sufficient malic acid to reduce spore germination and hyphal growth. Similarly, certain varieties of onion resist attack by the weak parasite, *Colletotrichum circinans*, because the dead cells contain protocatechuic acid which, diffusing to the surface of the outer scales, prevents spore germination (Walker *et al.*, 1929).

It has been shown recently that the washings from the surface of leaves from eleven of fourteen tree species contain substances which reduce the germination of *Botrytis cinerea* spores (Topps and Wain, 1957), and that substances excreted from a number of other plants stimulate or reduce the germination of spores of *Botrytis cinerea, Ascochyta pisi,* and *Puccinia triticina* (Kovacs and Szeöke, 1956). Rather surprisingly, *B. cinerea,* the least specialized of these pathogens, is most affected. Under natural conditions, substances such as these might accumulate and reach significant concentrations in films of moisture in which spores would germinate, particularly if they are excreted continuously.

In these and other examples, the ability of a particular pathogen to overcome the barrier which exists at the surface rests in its ability to grow in the presence of substances which reduce or prevent the growth of other organisms potentially pathogenic.

B. *Formative Effects at the Surfaces of Plants*

The question now arises as to whether or not plants secrete substances which in some way stimulate the germ tubes of spores to begin the process of infection. If such substances are present, the ability of a particular microorganism to infect a plant would depend on its ability to respond to them. Brown (1936) concluded that the stimulus to form appressoria and to penetrate the host is provided by the surface and is essentially nonspecific, and more recently Dickinson (1949) and others have emphasized the importance of the contact stimulus to obligate parasites. At present, there is no good evidence that the formation of appressoria, or other simple structures is conditioned by specific substances produced by the host plant. This is not to say that chemical factors have no influence in a more general way because Van Burgh (1950) has shown that nutrients stimulate formation of appressoria by *Colletotrichum phomoides* and Goode (1956) has shown that zoospores of *Phytophthora fragariae* encyst on living or dead roots of susceptible and resistant plants, but not on fine glass rods.

The relatively complex infection organs formed by some soil-borne pathogens have been little studied from the above point of view, but recently the stimuli affecting production of infection cushions by *Rhizoctonia solani* have been investigated. These structures consist of a roughly hemispherical mass of hyphae with the hyphal tips in contact with the cuticle. Kerr (1956) enclosed roots of radish, lettuce, and tomato seedlings in cellophane bags in soil infested with *R. solani,* and found that structures resembling infection cushions were formed on the surface of the cellophane enclosing radish or lettuce, but not tomato roots. Kerr and Flentje (1957) also found that the formation of infection cushions on radish roots is stimulated by substances diffusing to the surface from the underlying cells, and that only the radish strain responds to these substances. This is not the only stimulus because cushions are not formed when hyphae were in contact with cortical cells exposed by removal of the epidermis. On the living host it was postulated that contact with the cuticle as well as specific substances condition the formation of cushions. In contrast, Shepherd (1957) found no evidence that crucifer and lettuce strains are stimulated chemically, and considered that the stimulus is connected with the structure of the cuticle citing the findings of Mueller *et al.*, (1954) that the cuticular surface differs greatly in different species.

Clearly, the responses leading to cushion formation, and, therefore, to infection, may differ for different host-parasite combinations even for a single species of pathogen. When chemical stimuli are necessary, the ability of a pathogen to infect will depend on its ability to respond by the formation of cushions, and if the response is specific it has a certain survival value because only plants which the pathogen could parasitize are invaded.

III. Penetration of Intact Surfaces

A. *Nature of the Cuticle*

The epidermis of the shoot system, but not of roots, is covered by a well-defined layer, the cuticle, through which the infection hyphae of many pathogens have to pass before living cells of the host are reached. In plants in which it is well developed, the cuticle has a relatively complex nature consisting of a framework of cutin in a matrix of waxy materials which are extruded as a mass of rodlets on the outside. On the inside, the cuticle merges into a layer of pectic materials which further inward becomes the matrix for the cellulose of the outer wall of the epidermal cell. The waxy substances of the cuticle consist largely of higher alcohols and sometimes the corresponding ketonic and hydroxy acids are also present, particularly in young leaves. Cutin contains polymerized dicarboxylic and hydroxy carboxylic acids. The outermost layer of the cuticle is considered to be the most oxidized and polymerized and to contain the highest proportion of wax. A cuticle covers all aerial parts of the shoot system, and layers chemically very similar have been identified on the free surfaces of mesophyll cells, and on the inner walls of the epidermis exposed to the air spaces. In carefully prepared sections, the outer epidermal wall is often seen to be traversed by a series of very fine parallel lines; it has been suggested that these are the channels along which the materials which form the cuticle migrate to the exterior. They will be referred to later because they might have special significance in the process of infection.

Because of technical difficulties, much less is known about the nature of thinner cuticles which are the type most often penetrated by pathogens; in the following discussion it will be assumed that essentially they are as described above.

B. *Penetration of the Cuticle*

The penetration of the cuticle and outer wall of the epidermis by germ tubes coming from fungal spores has been studied in great detail by many workers. Particularly clear descriptions are available for almond leaves and *Clasterosporium carpophilum* (Samuel, 1927); apple and pear leaves and *Venturia inaequalis* and *V. pirina* (Wiltshire, 1915; Nusbaum and Keitt, 1938); *Vicia faba* and *Botrytis cinerea* (Blackman and Welsford, 1916); potato leaves and *Phytophthora infestans* (Pristou and Gallegly, 1954) among many others. The essentials of the process are similar in each. The germ tube becomes attached in some way to the cuticle; a flattened structure, the appressorium, also adherent to the surface, is formed by some fungi, and from this, or the germ tube, arises a small protuberance which grows into a style-like structure. This grows normally to the surface and penetrates the cuticle as an infection thread. After passing through the cuticle, and the outer layer of the epidermal wall, the infection thread increases in diameter to that of normal hyphae. At this stage, the barrier presented by the cuticle has been passed.

The aspect of this process which has attracted the attention of pathologists for many years is whether or not penetration of the cuticle and cuticularized layer of the epidermal wall depends on, or is facilitated by, substances produced by the pathogen. Otherwise, penetration must be wholly mechanical. Some of the facts relevant to this problem are as follows. The germ tubes of many fungi are able to penetrate intact films of materials such as paraffin wax, collodion, or gold where it can be assumed that chemical degradation plays no part. There is no penetration if such films exceed a certain thickness, hardness, or both. Also, Brown and Harvey (1927) showed that the epidermis of leaves of *Eucharis* spp. is penetrated readily if the leaf was plasmolyzed, but not otherwise; this is not easily understood if the cuticle is degraded chemically. Young leaves are more readily penetrated by many pathogens than are old leaves in which the cuticle is thicker and tougher, and it has been shown that the resistance of leaves of *Berberis* spp. to attack by the basidiospores of *Puccinia graminis* is positively correlated with the resistance of the cuticle and outer epidermal wall to penetration by a mechanical device (Melander and Craigie, 1927). More generally, the structures produced by germ tubes seem to be particularly well adapted for mechanical penetration. They become attached to the cuticle by a mucilaginous sheath, are often modified to increase the surface in contact with the cuticle, and the thrust on the attached hypha is reduced by the fineness of the infection thread, the diameter of which is often near the limits of resolution of the ordinary light microscope (Nusbaum and Keitt, 1938). No one has demonstrated that plant pathogens are able to degrade the cuticle chemically, but apparently plant pathologists have not made serious attempts to obtain fungal preparations able to do this. It is clear that cuticular materials returned to the soil are degraded by microorganisms; it should not be too difficult to isolate such organisms by enrichment techniques.

In the light of such evidence the view has been taken (Brown, 1936) that chemical degradation plays no part in the penetration of the cuticle by fungi. Against this view is certain cytological evidence for degradation. The fungus *Venturia inaequalis* is particularly suitable for this type of study because in the early stages of infection it is confined between the cuticle and the cellulosic part of the epidermal wall. Wiltshire (1915) stated that the cuticle is always thinner above hyphae and that the hyphae grow in the cuticle; this was taken as evidence for the degradation of cuticle, but Nusbaum and Keitt (1938) did not confirm this. Chaudhuri (1935) has also claimed that the germinating spores of *Colletotrichum gloeosporioides* degrade the cuticle of orange leaves, but here, too, the evidence is indirect and not conclusive. In studies such as these it is clearly important to distinguish between growth in and degradation of the cuticle itself and growth in the layers immediately beneath, which contain materials known to be degraded by many microorganisms.

At present, therefore, the evidence is that penetration of the cuticle is mechanical and does not depend on substances produced by the hyphae of invading organisms. The fact that plant pathogens do not degrade one of the most important barriers to infection poses a number of interesting questions, and, in particular, why an enzyme system able to do this has not been evolved. It is possible that as long as the fungus is able to penetrate the plant by other means, secretion of cuticle-degrading enzymes might be disadvantageous especially if the cuticle were thin, because the action of these enzymes would not be confined to the point of entry. Extensive degradation about this point would destroy the impermeability of the leaf surface to water and expose the young hyphae of the pathogen to desiccation. It would also provide a point of entry for secondary invaders, particularly bacteria, which, although unable to penetrate the intact cuticle, might be able to compete successfully with the primary pathogen at the surface of tissues underneath.

The fact that a fungus has the mechanical ability to penetrate the cuticle of a particular plant does not mean necessarily that it will do so because in ways already described the underlying cells might influence conditions in the surface film of water in which the fungus is growing. There may also be present in the cuticle substances which affect the growth of infecting hyphae. Martin *et al.* (1957) extracted from the ether washings of apple leaves a number of substances which, applied as sprays to apple leaves, prevented or reduced the germination of spores of *Podosphaera leucotricha;* some of these substances were also effective against *Botrytis fabae* on *Vicia faba.* How widespread such substances are in plants, and how effective they are in preventing infection remains to be determined. Unless there are such substances on or in the cuticle, and because the cuticle of many species of plants will be of the same order of hardness and thickness, it must be assumed that fungal hyphae often penetrate plants which they are unable to parasitize. There is little information on how often this happens because evidence of this sort is seldom looked for, and is not easily obtained. Johnson (1932) has, however, pointed out that *Colletotrichum circinans,* commonly thought to be typically a parasite of *Allium* spp., penetrates the leaves of a number of other species under greenhouse conditions, and Webb (1949) has shown that *Plasmodiophora brassicae,* regarded as a very specialized parasite confined to the Cruciferae, gets into the root hairs of plants belonging to a number of other families.

Before leaving this subject it must be pointed out that knowledge of the structure of the cuticle is still relatively limited and that no studies of penetration have been made with the techniques which have become available during the past few years. It is commonly supposed that the cuticle forms a continuous, relatively impermeable layer over the whole of the shoot. But Roberts *et al.* (1949) has shown that channels containing pectic substances traverse the cuticle of apple leaves. These channels are visible by ordinary light. The question arises as to whether or not there are much finer channels from the outside to the non-cuticularized part of the epidermal wall. If there are, and if they are occupied by materials easily hydrolyzed by fungi, penetration could be accomplished chemically. The probability that this happens is not high, but it must be remembered that the infection hyphae of some fungi are extremely fine and are, indeed, at the limit of resolution with ordinary light.

C. *Behavior of Wound Parasites*

A number of weak pathogens unable to penetrate unbroken cuticularized surfaces are able to parasitize the tissues beneath after entering through wounds. This is not necessarily because the spores do not grow at the surface because often there is no penetration even if the fungus is provided with nutrients which will ensure such growth. This means that these fungi lack the ability to produce the infection mechanisms produced by many of the shoot infecting fungi. They should, therefore, be unable to penetrate nonliving membranes. No systematic study along these lines seems to have been made, although the weak pathogens *Penicillium glaucum* and *Rhizopus nigricans* are less efficient in this respect than *Botrytis cinerea* which readily enters undamaged tissue (Brown and Harvey, 1927).

IV. INVASION OF UNDIFFERENTIATED CELLS

Under this heading will be considered the invasion of cells having living protoplasts and thin, undifferentiated cell walls, i.e., epidermal cells of roots and of shoots within the cuticle, mesophyll cells of leaves, and parenchyma which is exposed naturally or has become exposed by wounding. Because it presents special features, the behavior of obligate parasites will be considered first.

A. *Obligate Parasites*

The characteristic feature of the obligate parasite for this discussion is that, living on plant tissue, this type of parasite grows and multiplies only when associated with living cells of an appropriate host. If the host tissue is killed, death of the vegetative hyphae of the parasite follows quickly. In this way one distinguishes these parasites from fungi such as *Phytophthora infestans* which may not kill parasitized cells for some time, but which can also live on dead cells.

A few obligate parasites are wholly intracellular or wholly intercellular, but the majority are inter- or extracellular with short side branches, the haustoria, which penetrate the host cell. Haustoria are characteristic of obligate parasites, but are also found in a number of other specialized fungi. They are formed in much the same way as are infection threads which penetrate the cuticle of the shoot. Little is known about the factors which affect their formation, and how they function.

Once established, the hyphae of obligate parasites often grow freely between cells. This could be done mechanically, but recent work suggests that growth of rust hyphae between cells may be facilitated by chemical degradation of the wall. Van Sumere *et al.* (1957) have shown that extracts from uredospores, or germinating uredospores, of *Puccinia graminis* f. *tritici* contain enzymes degrading hemicelluloses, cellulose, and pectin. The pectic enzymes are produced only by germinating spores, and when pectin is present in the substrate. However, cytological studies have made it clear that degradation of the cell wall is limited in extent and it will be shown later that extensive enzymatic degradation of the cell wall generally kills the protoplasts; this itself would prevent growth of an obligate parasite.

Tissues successfully colonized by obligate parasites show little sign of damage for some time although there is a heavy intercellular growth of hyphae, and many of the cells are penetrated by haustoria. Whereas it is unlikely that the host cells derive much benefit from the parasite, the good growth of the pathogen shows the extent to which the nutrients of the host are being used. The accumulation of C^{14} compounds at the sites of infection of cereal leaves by *Puccinia graminis* and *Erysiphe graminis* has been demonstrated by Shaw and Samborski (1956) who suggested that the metabolism of the host tissue near the lesion is stimulated by substances produced by the pathogen, the host cell, or both. Clearly if there were no stimulation and mobilization of nutrients, growth of the pathogen would be at the expense of invaded cells, and the starvation of these cells might lead to their early death, and to that of the pathogen as well. The ability to increase the metabolic rate of host cells might, therefore, be a factor in successful parasitism.

In hosts which are not freely parasitized by an obligate parasite, resistance is expressed in a number of ways, but often the stage is reached at which a haustorium is put into a host cell which then dies, more or less quickly, depending to some extent on the environment, but more importantly, on the particular host-pathogen combination. The parent hypha may continue to produce more haustoria which behave in the same way until the reserves of the pathogen are used up. The pathogen then dies after having killed a small group of cells. The way in which the cells are killed is not known. It could be by toxins produced by the pathogen. These would have to be very selective in their action because often the cells of a closely related variety of the same species are not killed. The host cells may not provide essential nutrients, and this may interfere with the metabolism of the pathogen so that toxic by-products accumulate. Whatever the reason, the pathogen fails to establish the compatible relationship which it achieves with a susceptible plant, and, therefore, dies.

This type of pathogen, when successful, is able to extract nutrients from host cells without killing them. This is not to say that the metabolism of the invaded cell, and neighboring cells, is not altered. There is, in fact, much good evidence that it is substantially changed in ways which will be described later. Sooner or later, these changes affect host cells because the benign relationship between host and pathogen comes to an end. Then there begins a series of degradative changes ending in the death of the cell, not, however, before the pathogen has grown extensively and sporulated.

The barrier to infection presented by a plant resistant to an obligate parasite is, therefore, the susceptibility of the cytoplasm of the host cell to the metabolic products of the fungal cell living in close association with it, in the case of fungi such as *Plasmodiophora brassicae*, in the cytoplasm itself. It is not difficult to see how such barriers could arise, but it is more difficult to envisage how obligate parasites overcome them, particularly because the balance between compatible and incompatible relationships is often so delicate and liable to be upset by slight changes in the metabolic state of the host. The nature of these relationships, especially where the cytoplasm of pathogen and host is not separated by any easily visible barrier, presents one of the most baffling and challenging problems in pathology.

Although not obligate parasites in the strict sense, the smut fungi have some interesting features in this connection. Typically, but not always, a seedling is infected and hyphae with haustoria become distributed throughout the entire host. The growth of the host is not greatly affected, and there are no obvious deleterious effects on host cells next to hyphae. At this stage, the parasitism of the smuts depends upon an ability to live compatibly with host cells. But this relationship is broken later because in ways characteristic for different diseases, a large part of the host tissue is killed and becomes occupied by a mass of teliospores. During this process the cells of the host lose their identity and, presumably, their contents are used in the formation of the spores. Generally, the change occurs in tissue anatomically and physiologically different from that in which the fungus has grown vegetatively, but occasionally spores are formed in parts of the shoot system which are probably not very different physiologically from parts in which growth is vegetative, e.g., *Ustilago bistortarium* which is perennial in the rhizomes of *Polygonum bistortoides* grows systemically through the plant but produces spores only in a narrow band at the edge of the leaf (Fischer, 1953). Just before the formation of teliospores, it must be assumed that the fungus produces extracellular enzymes which are able to degrade the host tissue extensively; in the absence of damage during vegetative growth such enzymes are not active earlier. This aspect of pathogenicity of smut fungi has been little studied. In view of the general similarity of rust and smut parasitism and the ability of smut fungi to grow on artificial media this might well repay investigation because their potentiality for producing extracellular enzymes could be easily studied.

B. *Types of Barriers Present in Plants*

1. *Barriers Caused by the Absence of Essential Nutrients*

It has often been suggested that obligate parasites cannot grow on dead tissue of a susceptible host because only the living cells are able to supply the nutrients essential for growth. Similarly, plants may be resistant to obligate parasites because they do not contain such nutrients. Garber *et al.* (1956) and a number of earlier workers have proposed that similar factors apply in parasitism by nonobligate types. Thus, mutants of *Erwinia aroideae*, deficient for certain amino acids, were less virulent than the wild type (Garber *et al.*, 1956), but prototrophic reversions from an avirulent mutant deficient in arginine had the original virulence. In similar work with *Venturia inaequalis* and apple leaves (Keitt and Boone, 1954; Kline *et al.*, 1957) virulence was restored when the required nutrilite was added to the inocula of six out of eight different mutants. Here it may be assumed that one barrier to infection is the absence of an essential substance in available form. The last qualification is necessary because Kline *et al.* (1957) found that each of the eight deficient mutants grew on juice from apple leaves.

This concept applied to absolute deficiencies for one or a small number of substances is probably of limited value in attempting to understand mechanisms of pathogenicity because, obviously, a pathogen can parasitize only those tissues which provide materials essential for its growth. Also, apart from the fact that most plant pathogens other than obligate parasites have simple nutritional requirements, the tissues of higher plants almost always contain the substances for which deficiencies in some fungi have been reported. Sometimes, of course, growth of a pathogen in a host may be limited by the concentration of a substance for which the pathogen is deficient. This, however, is only another aspect of the general rule that for continued parasitism the host must provide materials for growth in suitable proportions.

On the other side of the picture, the ability of an organism to grow when supplied only with simple nutrients is not likely to contribute much to its pathogenicity. In fact, many saprophytes and weak pathogens fall into this category.

2. *Resistance Caused by the Presence of Inhibitory Materials*

Here the plant is considered to be resistant because it contains substances which reduce or inhibit growth of some organisms. Pathogens of such plants either are not affected by the inhibitory substances, or else possess mechanisms which render them ineffective.

This type of barrier to infection has always attracted a good deal of attention, probably because it is one of the easiest to visualize. There are numerous examples in the literature which provide circumstantial evidence for such barriers. As a group, phenolic substances have been studied from this point of view for many years. Recently, Johnson and Schaal (1952, 1957) showed that the phenols, particularly chlorogenic acid, present in the outer layers of potato tuber are important in resistance to infection by *Streptomyces scabies*, and Kirkham (1957) in similar work with *Venturia inaequalis* and *V. pirina* attacking apple and pear leaves, considered that phenols in the leaves play some part in the patterns of resistance shown in these diseases although it was emphasized that they are not the only factors involved.

The different alkaloids have a restricted distribution among plant species, and some workers have produced evidence that they are important in disease resistance. Thus, McKee (1955) attributed the resistance of cells at the surface of wounds in potato tubers to the solanine present in the cell vacuoles, and the resistance of some plant species to infection by *Phymatotrichum omnivorum*, a pathogen with a very wide host range, is thought to depend on their high content of certain alkaloids (Greathouse and Rigler, 1940).

On the other hand, the presence of a substance in plant tissues at concentrations which would be toxic *in vitro* does not necessarily mean that this substance acts as a barrier *in vivo*. An example of this is provided by the work which has been done on the chemical basis of resistance in the Cruciferae to *Plasmodiophora brassicae*. Rochlin (1933) claimed that *Brassica nigra* is resistant because of its high content of mustard oil which occurs in undamaged cells as a glycoside, and is released by the β-glycosidase activity of damaged cells. But it has been shown that highly resistant and highly susceptible lines of black mustard have about the same amount of mustard oil and glycosidase, and that a resistant line, grown so that it contained little mustard oil, nevertheless retains its resistance (Stahmann *et al.*, 1943; Pryor, 1940).

It is, in fact, rather remarkable how few cases there are in which resistance of a plant to a particular pathogen can be traced to the presence in the plant of specific inhibitory substances. There are a number of reasons for this. The substances may not be present in the parts of the plant which are colonized by the pathogen, and in invaded tissue they may not be present in an available form. In this connection, the fact that obligate parasites, and some other fungi, do not kill host cells would be important because some toxic substances may be released only on death of the host cell. By inducing pH changes in invaded tissue, the pathogen might reduce the permeability of its cells to the toxic substances. Products of the metabolism of the pathogen may also react with the toxins to form innocuous compounds. A special case of this sort is described by Bazzigher (1955, 1957) for *Endothia parasitica* and certain other fungi which produce adaptive enzymes which degrade tannins and phenols. If such substances are toxic to some fungi, an ability to degrade them would be a detoxification mechanism, although in this example it would be difficult to show that the fungi used are not intrinsically tolerant of these toxins.

The pathogen may become adaptive in ways which have been described for bacteria and drugs, and fungi and fungicides. For example, Agerburg *et al.* (1933) showed that the alkaloid solanine present in tomato leaves has less effect on the germination of spores of *Cladosporium fulvum* obtained directly from infected leaves than on conidia obtained from cultures. This might be an instance where exposure of a pathogen to a toxin either causes phenotypic and genotypic changes, or leads to the selection of cells having greater resistance.

3. *pH and Osmotic Pressure Effects*

Microorganisms which grow well only over a narrow pH range are probably restricted in their parasitism by this factor. For example, most bacteria and some fungi probably do not parasitize fruits such as apples because the tissue has a low pH. Cole (1958) has shown that when apples are invaded by certain bacteria there is little spread of the pathogen from the point of inoculation, the damage being produced by toxins which diffuse into the tissue. Horne (1932) suggested that the increased susceptibility of overripe apples to attack by a number of fungi depends partly on the rise in the pH of the tissue.

Osmotic pressure effects in parasitism have been studied relatively little except in the obligate parasites. Obviously they are important because in order to grow, a pathogen must absorb water across a semipermeable membrane. Where the limiting membranes of host and pathogen are in intimate contact as in the haustoria of rusts, the pathogen will gain water only if the suction pressure of the hyphae exceeds that of the host cell; Thatcher (1939) has shown this to be true for two species of *Uromyces* on pea and carnation plants. Differences in osmotic pressure between the cells of host and pathogen may be intrinsic and present from the beginning, or may be induced by the action of substances produced by the pathogen which affect the permeability of the host cell. Such substances will be dealt with later.

Pathogens which cause extensive degradation of plant tissues might create difficulties for themselves by breaking down polymers such as starch and pectins, thus producing solutions of high osmotic pressures. This may explain the results of Johnson (1947) who showed that water-congested leaves are much more susceptible to attack than were normal leaves and those of Lapwood (1957) and Murant and Wood (1957) who found that parasitism of potato tubers by bacteria is greatly affected by the water content of the tissue, and that free water in intercellular spaces destroys the resistance of tubers to attack by organisms nonpathogenic to ordinary tubers.

The ability of an organism to develop high suction pressures or to reduce those of the host cells can, therefore, be considered a factor in pathogenicity.

4. *Agglutinating Substances*

There are in the blood sera of many species of animals living under natural conditions substances which react with bacteria in much the same way as do specifically induced agglutinins, bactericidins, or antitoxins. There is some evidence that substances with a similar action occur also in plants. Berridge (1924) has described some of the earlier work on this subject and has herself shown that the fresh juice from potato tubers agglutinates and plasmolyzes certain nonpathogenic bacteria but does not affect pathogenic species. In this instance, the ability to resist agglutination at the surface of a damaged tuber might be a factor affecting pathogenicity. But Manil (1936) did not find that resistance to *Bacterium tabacum* or *Pseudomonas syringae* is related to the agglutinating power of the sap, and Murant and Wood (1957) found that although two species of bacteria nonpathogenic to potato tubers are agglutinated in the way described by Berridge, and a pathogenic species is not, a third nonpathogenic species belonging to another genus also is not agglutinated.

Very little is known about the nature of the substances in plants which cause bacteria to agglutinate and, in particular, whether or not any of them have the complex structure of the corresponding substances present in some animals. And little is known about the ability of some bacteria to be unaffected by such extracts, for instance, whether or not some bacteria secrete substances which degrade the agglutinins or inactivate them in some other way. Substances, collectively known as "aggressins," with such properties are known from immunity studies in animals.

5. *General Nutrient Conditions in the Host*

Above have been described some of the ways in which substances initially present in plant cells form barriers to infection because they reduce or prevent growth of the invading organism. Similar effects may arise because the host tissue does not provide the pathogen with a substrate suitable for continued growth, and these effects can be simulated by growing fungi or bacteria on unsuitable artificial media. The organism starts to grow but the metabolism becomes unbalanced and one or more products which retard growth accumulate. This sort of process once started can rapidly become cumulative, can stop further growth, and may even kill the cells which have been produced. Some of the factors which affect the metabolism of an organism in this way are the C : N ratio of the substrate, the type of nitrogen source, trace elements, and growth factors. If the C : N ratio is high, as is often the case in higher plant tissues, sugars are used only partially, and incompletely oxidized products such as organic acids accumulate and may reach concentrations at which growth of the pathogen is reduced. An example of a trace element effect is that zinc deficiency unbalances carbohydrate metabolism and organic acids accumulate. Differential usage of different sources of nitrogen can lead to pronounced pH changes. Some fungi, e.g., certain Fusaria, are known to be affected by such changes. The ability of an organism to continue growing in the presence of these "staling products" may, therefore, be a factor contributing to their ability to parasitize a host tissue.

Along a rather different line, fungi are essentially aerobic organisms and probably can metabolize anaerobically only to the extent of their indigenous carbon stocks. In contrast, many bacteria, some of them plant pathogens, are facultative anaerobes, and are able to grow deep in tissues where the oxygen would limit aerobic respiration. In a large mass of tissue with a small surface in relation to volume, the rate at which a lesion advances may depend on the rate at which the pathogen can obtain oxygen at the advancing edge of the lesion, or on the ability of the pathogen to metabolize anaerobically. Although no precise data are available, some rots of storage tissue grow so rapidly that it is difficult to believe that there is sufficient oxygen at the edge of the lesion for aerobic respiration. In such cases the ability of an organism to grow anaerobically would free it from competition by secondary invaders which do not possess this property. Also, tolerance of the by-products of anaerobic metabolism would contribute to pathogenicity because in sufficiently high concentrations, these substances might kill host cells in much the same way as cells are killed by their own anaerobic metabolism, e.g., brown heart of apples.

Another factor is the suitability of the host for the production by pathogens of toxins, and the extracellular enzymes which degrade plant cells. It will be shown later that the nutritional requirements for production of these substances are often rather critical and not always the same as those which support good vegetative growth.

6. *Cell Walls as Barriers*

Many fungi which successfully invade higher plants have little or no effect on the cell walls. Although with obligate parasites such as the rusts and with a few facultative saprophytes the cell wall may be penetrated by haustoria, this is probably done mechanically and it has little effect on the general structure. Because resistance in such cases is generally associated with death of the cells, it is not surprising that parasitism is not accompanied by breakdown of the cell walls.

Whether or not bacteria are able to parasitize plant tissue without degrading cell walls is rather doubtful. Bacteria are recorded from healthy tissues showing no damage to the cell walls but the bacteria are probably dormant and inactive until conditions in the tissue become suitable for their multiplication.

In many other diseases the pathogen kills the cells of the host in one of a number of ways and lives on moribund or dead cells. Frequently, the cells of the host tissue are killed in a zone in front of the pathogen, and in this zone there is often clear evidence of cell-wall degradation. In such lesions, often characterized by a mass movement of the pathogen along a broad front, the cell wall probably functions as a barrier to infection in a number of ways. In the first place it is composed largely of insoluble materials and may be of considerable thickness relative to the dimensions of the pathogen. It therefore presents a complete barrier to organisms such as bacteria which are unable to penetrate it mechanically. Although many fungi penetrate cell walls mechanically, this is done by infection threads many times narrower than normal hyphae so that hyphae penetrating successive cell walls would have numerous constrictions along their length; this, presumably, would interfere with the flow of materials along the hyphae. Furthermore, the action of the pathogen on host cells depends upon movement into the cells of extracellular enzymes and other substances produced by the pathogen, and movement out of the cells of nutrient materials. Although materials are interchanged between adjacent cells through the cell wall as well as through the plasmodesmata, presumably, substances would move across cell walls much more rapidly if they were degraded. It is also possible that protoplasts are killed if certain structures in the cell wall are substantially altered. Degradation of the cell wall could, therefore, be a way in which pathogens kill host cells.

At a later stage in this sort of parasitism, the cell wall may be an important source of energy for organisms able to degrade the constituent polymers. The chemical ability to degrade cell walls is, therefore, one of the most important attributes of the nonobligate type of parasite, and for this reason it will be considered in some detail in the next section.

C. *Pathogenic Mechanisms of Microorganisms*

1. *Enzymes Degrading Cell Walls*

The cell wall consists of a meshwork of cellulose microfibrils embedded in a matrix which in parenchyma cells contains pectic materials as the main constituents with lesser amounts of arabans, galactans, xylans, and hemicelluloses. The primary walls of adjacent cells are separated by a middle lamella consisting largely of pectic substances. The only point that need be made about cellulose is that although it is considered to consist essentially of linear chains of glucose in β-1,4-glycosidic linkage, it is suspected that other linkages between glucose units and also a small number of reactive groups are present. These linkages and groups could profoundly affect the enzymatic degradation of the chain, and the relationships between cellulose and other components of the cell wall.

The essential structure in pectic materials is a linear chain of anhydrogalacturonic residues in α,1,4-glycosidic linkage. If all the carboxyl groups on carbon 6 are free, the substance is pectic acid with pectates as salts. Some of the carboxyl groups may be esterified with methyl alcohol to give pectinic acids with pectinates as salts; pectins are pectinic acids with a high proportion of the carboxyl groups esterified. Pectic acid and salts with polyvalent cations are insoluble; pectinic acids are soluble but with decreasing methoxyl content there is an increasing tendency to form insoluble gels.

The different properties of the pectic materials of the middle lamella, and the primary and secondary cell walls probably depend primarily on differences in degree of esterification. In the middle lamella there is little esterification and the pectic material is considered to consist essentially of a firm, hydrophobic meshwork of polyuronide chains extensively cross-linked through the formation of calcium and magnesium salts. In the primary and secondary cell wall the higher degree of esterification reduces cross-linking and a plastic and relatively hydrophilic meshwork is formed. The insolubility of this type of pectic material, commonly referred to as protopectin, is still conjectural. It may primarily be a matter of chain length and cross-linkage between adjacent chains.

Little is known about other materials in cell walls and about the enzymes which degrade them. In parenchyma cells they are only a small proportion of the materials present, but it would be rash to dismiss them as unimportant because their presence, even in small quantities, could profoundly alter the susceptibility of the main components to attack by enzymes.

Enzymes degrading cell walls are known almost exclusively from bacteria and fungi. The cellulases degrade cellulose and its derivatives. In spite of intensive study, their mode of action is not known with certainty. One view is that there is a single enzyme which by random cleavage converts cellulose to glucose (Whitaker, 1957). Another states that a second enzyme, cellobiase, is required for the conversion of cellobiose to glucose (Aitken *et al.*, 1956), whereas Reese (1956) considers that cellulose is degraded completely only by the successive action of a number of enzymes; the first, designated C_1 converts native cellulose into soluble straight chains of anhydroglucose residues which are then degraded by a second enzyme C_x which hydrolyzes the β-1,4-glycosidic linkages to produce cellobiose, this then being converted to glucose by the action of a β-glucosidase or cellobiase.

Whatever the method of degradation, it is known that a number of plant pathogens can degrade cellulose in one or another of the manufactured forms to produce cellobiose or glucose. Almost always, cellulase is produced *in vitro* only when the specific substrates are present in the media.

2. *Action of Cellulolytic and Pectic Enzymes in Vivo*

The pectic enzymes are more diverse than the cellulases in mode of action and properties. They may be classified as follows. Pectinesterase deesterifies pectinic acids to give pectic acid and methyl alcohol. Polygalacturonases rupture the glycosidic linkages between residues. There are now known to be a number of polygalacturonases for which Demain and Phaff (1957) have proposed the following nomenclature: endopolygalacturonase hydrolyzes pectic acid or pectates by random splitting, exopolygalacturonase degrades similar substrates but preferentially attacks terminal linkages; endopolymethylgalacturonase attacks pectinic acids by random splitting, and exopolymethylgalacturonase attacks terminal linkages. In addition, there may be other enzymes which attack the main chain; these will be referred to later.

In the early stages of infection of parenchyma there is little microscopic evidence of cellulose degradation although the tissue has lost most of its coherence. Cellulases have not been looked for often in rotted tissues, but in some cases they are known to be absent, or present in very low concentrations. Cole (1958) was unable to extract cellulases from apples rotted by any of a number of fungi, and the author has found the same for potato tubers rotted by *Erwinia aroideae*. But Husain and Kelman (1957) state that juice from the tissue of tomato plants infected by *Pseudomonas solanacearum* possesses cellulase activity although it is not high, and the substrate used in the tests was a soluble derivative.

Although many plant pathogens produce a cellulase *in vitro*, most workers measure activity in terms of an ability to degrade salts of carboxymethylcellulose which are soluble and therefore facilitate experimentation. It is known that some organisms which degrade soluble derivatives attack insoluble and relatively unchanged cellulose only slowly if at all (Reese *et al.*, 1950), so that the ability to degrade such derivatives may be little related to an ability to degrade the cellulose of the cell wall. Furthermore, the author has found that filtrates from cultures of *Myrothecium verrucaria* which does degrade insoluble cellulose, does not macerate thin slices of plant tissues, although this happens readily with filtrates from other organisms having no cellulolytic activity. At present, there is no good evidence that cellulases are important in the early stages of infection by plant pathogens. This may be because at this stage the substrate is separated from the pathogen and its secretions by the matrical substances between the fibrils, and that by the time the fibrils have been exposed, much of the damage to the cell has been done. Also, cellulases are almost always produced *in vitro* only when the specific substrate is present and often, production and activity are reduced by glucose and other sugars. At the beginning of lesion formation, conditions would, therefore, seem not to be very favorable for cellulase secretion. But cellulase may be produced later when dead tissue is degraded and the gradual accumulation of sugars which would follow might have important effects on the physiology of the pathogen particularly in the formation of reproductive structures. It is also probable that much of the degradation occurs through the activity of secondary invaders, or after the dead tissue has been returned to the soil. It may be significant that most fungi which damage cotton fibers in the field are either nonpathogenic or very weakly pathogenic to higher plants.

The ability to degrade cellulose does not seem to be an important way of breaking down the barrier of the cell wall, at least not in rapidly spreading lesions, probably because the enzymes are not formed quickly enough, and because their action is too slow to be useful to the fungus or bacterium in the actively pathogenic phase. These enzymes may be more important in slowly developing diseases where the pathogen is associated with the host for relatively long periods as in some vascular wilts, and in diseases in which the host material is extensively used after the tissue has been killed, e.g., dry rot of potatoes caused by *Fusarium coeruleum*.

Although it is difficult to assess the importance of cellulases at the present time, the facts are that many pathogens do produce cellulases and grow with insoluble cellulose as the sole carbon source, so it is very likely that under some conditions, cellulases are produced *in vivo* and play a part in pathogenesis.

In contrast to the uncertainty about the role of cellulases, there is abundant evidence that pectic enzymes are often important from the beginning. In soft rots the cells separate along the line of the middle lamella; this implies the degradation of a layer composed largely of pectic materials. In later stages, loss of pectic substances from cell walls may be shown by staining or, better, by analyses of sound and rotted tissue (Cole, 1958). Culture filtrates containing pectic enzymes produce in living tissues many of the effects observed *in vivo*, whereas filtrates not containing these enzymes do not. And pectic enzymes, not of host origin, can be isolated readily from host tissues invaded by certain plant pathogens (Wood and Gupta, 1958). Many pathogens produce pectic enzymes in artificial media and in extracts of host tissues; of a large number of pathogens investigated by the author, only *Phytophthora infestans* failed in this respect. This widespread ability to produce enzymes which degrade plant tissues, itself suggests that pectic enzymes are important agents in breaking down the cell wall barrier.

In most soft rots one of the earliest disease symptoms is separation of cells along the middle lamella, often in advance of the pathogen. Because this layer is believed to consist largely of pectates, it would be degraded by exo- and endopolygalacturonases; the gel structure is lost, and lower molecular weight compounds and, finally, galacturonic acid are produced.

The author has observed that enzyme preparations which rapidly disintegrate slices of parenchymatous tissue macerate thin slices of meristematic tissue such as root tips very slowly. This may be caused partly by the absence of intercellular spaces so that the middle lamella is not as accessible as it is in parenchyma. But it is more probable that the middle lamella of very young and of mature cells differs in composition and that in meristematic cells it is more resistant to degradation by pectic enzymes. In this connection, the recent work of Ginzburg (1958) may be significant because evidence was obtained that the middle lamella between the cells of pea root tips contained protein which retards maceration caused by calcium sequestering agents. If protein were present, the ability to degrade the middle lamella would depend on the secretion of proteolytic enzymes which would expose the pectates to the action of pectic enzymes.

Generally, it has been assumed that the middle lamella is of more or less constant composition and structure. If there were differences in different parts of a plant, and between plant species, some of the differences in susceptibility to attack by pathogens could be accounted for.

The pectic substances of the primary and secondary walls which yield pectinic acids of high methoxyl content and high molecular weight on mild acid hydrolysis, are also readily brought into solution by the enzymes of a number of pathogens. The way in which this is done initially is not known because the reasons for the insolubility of protopectin are not known; but at a later stage, when the materials, although still of very high molecular weight, are soluble, further breakdown may occur in a number of ways. If pectinesterase, produced by the pathogen or by the host, is present and active, pectinic acids may be degraded by exo- or endopolygalacturonases. Otherwise they would be degraded by exo- or endopolymethylgalacturonases.

From numerous studies which have been made in the past few years (Wood, 1955; Demain and Phaff, 1957), it is clear that microorganisms may degrade pectic substances *in vitro*, and presumably *in vivo* too, in a number of ways. Also, pectic enzymes having the same mode of action may differ in their physical and chemical properties, and particularly in their reaction to pH. Pectic enzymes generally have sharp peaks of activity at certain pH values so that the initial pH of a tissue, and the changes in pH caused by the pathogen, may be important in this respect. There is, however, some evidence that pathogens may possess certain adaptive mechanisms which reduce the importance of the pH factor. Filtrates from cultures of *Erwinia aroideae* on synthetic media have a pH optimum of 8.0 or higher, and activity diminishes rapidly below the optimum. But this organism readily rots potato tubers where the pH at the surface of damaged tissue and of the juice of rotted tissue is about 6.2. If the organism is grown in potato sap, the enzyme is more active at pH 6.0 than at higher values, so that there are differences in the properties of the enzymes produced under different conditions (Murant and Wood, 1957).

There is also evidence that an organism able to produce more than one type of chain splitting enzyme produces different proportions of these enzymes under different conditions. Cole (1958) found that filtrates from cultures of *Penicillium expansum*, active on pectinates and plant tissue, degrades pectates very slowly, but extracts from rotted tissue, also active on the first two substrates, rapidly degrade pectates as well. Here, therefore, *in vitro* experiments alone would have given a misleading picture of the activity of the pathogen in apple fruit.

Apart from an intrinsic ability to secrete pectic enzymes, secretion may also be much affected by the substrate. Some organisms produce chain splitting enzymes constitutively, others produce them only if the specific substrate is present, whereas others produce them more abundantly under these conditions. Pectinesterase seems to be more often produced adaptively than the chain-splitting enzymes. That these factors may be important in infection is shown by the work of Green (1932), who showed that *Penicillium digitatum* and *P. italicum* do not invade citrus fruits if certain nutrients supporting good growth are added to the inoculum, but do so if orange juice is added. Almost certainly the juice would have contained pectic substances which probably stimulate the formation of the pectic enzymes necessary for infection.

The C : N ratio of the substrate can also greatly influence enzyme production, high ratios generally reducing secretion. This could be significant in tissues with high carbohydrate content. A readily available carbon source would promote good growth of the pathogen, and this would use up the nitrogen which would not be available, therefore, for the production of the pectic and other enzymes needed for the disintegration of the tissue unless the organism were able to utilize its own reserves for this purpose.

More specific factors may also affect secretion of pectic enzymes. Extracts from some plants reduce or prevent secretion of enzymes in media otherwise suitable (Singh and Wood, 1956). Byrde (1957) has shown that the resistance of the fruit of some apple varieties to attack by *Sclerotinia fructigena* may be attributable to their high content of substances which reduce pectic enzyme activity. Cole (1958) found that a pathogen itself may alter the activity of the pectic enzymes it has secreted. In a comparative study of the rots of apples caused by different fungi and, in particular, the firm, brown rot produced by *Sclerotinia fructigena* and the soft, white rot caused by *Penicillium expansum*, it was found that there is a much greater loss of pectic materials in the white rot and that only in this rot are chain splitting enzymes present. With these differences is associated a much greater loss of phenolic substances in the firm rot. The polygalacturonase of *S. fructigena* is inactivated after incubation with the products formed by the oxidation of certain phenols present in apple tissue. There is little oxidation in the white rots so that the inhibitory product is not formed, and continuous degradation of the pectic materials causes a greater loss of pectic materials from the cell walls. The reason for the absence of oxidation in white rots has not been determined, but is possible that *P. expansum* produces inhibitors of host oxidative enzymes, and, additionally, does not itself oxidize phenols.

In this case, therefore, the pathogen breaks a barrier by inhibiting an enzyme system. If this system were not inactivated it would produce inhibitors of enzyme systems of the pathogen which are essential for pathogenicity.

The production of inhibitors in the brown rot does not seem to have much effect on the pathogenicity of *S. fructigena* probably because it is offset by the ability of this fungus to grow rapidly through the tissue. But similar systems may be responsible for the self-limiting type of lesion often produced in leaves. Here the phenolic content is often high, and the fungi have an intrinsically low rate of growth.

3. *Consequences of Cell Wall Degradation*

In a soft rot lesion many of the pathogenic effects are produced before the cell wall has lost much of its visible structure. At this stage the cells are dead, killing often occurring in advance of the pathogen. The cells may be killed by toxins produced by the pathogen; the movement of such toxins through the tissue, and through cell walls would be facilitated by the action of wall degrading enzymes. There is also a good deal of evidence that the action of pectic enzymes on the cell wall, or on protoplasmic structures closely associated with the wall, can itself kill the protoplasts.

Death of the cells is followed by a complex series of reactions, some autocatalytic in nature, others initiated by the pathogen. Under natural conditions, the course of these reactions is often complicated by secondary pathogens once the cellular resistance of the host is destroyed. Analysis of these processes is obviously a formidable task, but some aspects of it are relevant to pathogenicity. Thus, in many diseases the lesions continue to grow, but in others, the lesions stop growing soon after their formation. The nature of some of the barriers which intervene at this stage will be considered later.

4. *Production and Action of Toxins*

Obligate parasites obtain nutrients from living cells, and their parasitism depends on the fact that they do not kill host cells. In contrast, certain properties unique to living cells are barriers to infection by many other pathogens. The selective permeability of the living protoplasmic membranes obviously limits the action of some metabolic products of the pathogen, and also reduces the availability of materials within the cell. Furthermore, only living cells produce the defense mechanisms important in the resistance of some plants. An ability to kill host cells is, therefore, a way in which an important barrier to infection can be overcome. The killing of cells by pectic enzymes has already been considered. In certain other diseases cells are killed by relatively low molecular weight substances, generally referred to as toxins.

Pathologists have often been tempted to regard toxins as important in symptom production because fungi and bacteria growing on artificial media frequently produce substances toxic to plant cells. There is no reason why these or similar substances should not be produced, and kill cells, when the pathogen is growing on the host. In many diseases, symptoms appear in parts of a plant away from the pathogen; this can be interpreted as the action of substances which move from the site of infection. If a pathogen produces *in vitro* substances which can reproduce in host plants some of the natural symptoms, it is tempting to ascribe such symptoms to the action of toxins produced *in vivo*. It is, therefore, surprising that very seldom has it been shown unequivocally that specific toxins are important in pathogenesis although this has often been claimed, generally on inadequate evidence. In this connection, the paper of Dimond and Waggoner (1953a) is important because it emphasizes the proof required before a definite role in symptom production can be ascribed to metabolic products of a pathogen. Such substances, for which the term "vivotoxins" was proposed, must be found in diseased plants and must be able to reproduce some or all of the natural symptoms.

Apart from substances active in low concentrations, cells may be killed by concentrations of substances of relatively low toxicity which accumulate because the composition of the host tissue does not permit a balanced metabolism. Gibson (1953) considered that oxalic acid causes the lesions on the hypocotyls of groundnut plants infected with *Aspergillus niger*, and it may be significant that *Sclerotium rolfsii* which attacks the soft tissues of a large number of plants rapidly reduces the pH of many types of media to low values (Abeygunawardena and Wood, 1957).

There is now a good deal of circumstantial evidence that toxins are important in some necrotic diseases of leaves (Brian *et al.*, 1952; Braun, 1955; Pringle and Braun, 1957; Ludwig, 1957); in vascular wilt diseases caused by *Fusarium* spp. (Dimond and Waggoner, 1953b; Gäumann, 1951, 1957); and in diseases where there are pronounced formative effects (Yabuta and Hayashi, 1939a, b; Braun, 1954; Sequira and Steeves, 1954). A full account of these is given in Chapter 6 of Volume I and Chapter 9 of this Volume. Only the more general aspects of the action of toxins on plant cells will be considered here.

Microorganisms which readily change the pH of the medium in which they are growing to high or low values, often grow in the extreme conditions they produce. Cells of most higher plants probably function normally only over a relatively narrow pH range, and although extreme pH may not kill the cells immediately, prolonged exposure may do so (Overell, 1952; Gibson, 1953). Changes in pH could also reduce the permeability of host cells to toxins by altering the plasma membranes or by reducing the dissociation of the toxins.

Effects of a similar general nature may be produced by substances affecting oxidation-reduction equilibria in host cells, but there is little evidence available on this point at present.

The ability of some metabolic products to chelate with essential metals could produce toxic effects (Deuel, 1954). The toxins lycomarasmin and fusaric acid, are chelating agents and Gäumann and Naef-Roth (1954, 1955) have shown that these and other chelating agents have similar effects on tomato shoots. Certain amino acids and other organic forms of nitrogen are also well known as chelating agents. The secretion by some organisms of relatively large amounts of organic nitrogen compounds, may be a factor in pathogenicity because these substances cannot be used by the fungi which produce them (Morton and Broadbent, 1955).

At least one metabolic product of fungi is known which probably damages cells of higher plants by acting as an enzyme poison. Clavacin, produced by a number of species of *Penicillium* and *Aspergillus,* is highly toxic to the cells of higher plants, and Miescher (1950) has shown that it probably acts by blocking sulfhydryl groups of enzymes or essential metabolites. Little is known about the production by pathogens of enzyme poisons, but Brian *et al.* (1956) have shown that apples infected with *Penicillium expansum* contain large amounts of clavacin.

A toxicity which has been much studied in bacteria is that in which enzyme reactions in susceptible cells are inhibited by substances similar to the substrate of the enzyme. The inhibition is competitive when the toxin combines with the same active centers on the enzyme molecules as do the substrates and cannot afterward be metabolized, and is noncompetitive when the toxin combines with active groups on the enzyme and so makes others unavailable to the substrate. Toxins of pathogens may act in these ways, e.g., interference of the methionine metabolism of host cells by the toxin produced by *Pseudomonas tabaci* (Braun, 1955).

In some plant diseases, infection is followed by an increase in the respiration of the infected tissue not wholly accounted for by the respiration of the pathogen. Sometimes, the respiration of invaded tissue near infected tissue is also increased. This aspect of the action of toxins has been much studied in recent years particularly in obligate parasites, and it has been suggested that the increased respiration follows the action of toxins on the host cells (Hellinga, 1940; Millerd and Scott, 1957; Uritani and Akazawa, 1955).

However, Farkas and Király (1955) and Daly and Sayre (1957) obtained no evidence that toxins are responsible for the increased respiration.

Reduction of respiration in host cells by toxins has also been reported. Naef-Roth and Reusser (1954) found that fusaric acid reduces respiration in excised tomato shoots, and lycomarasmin and fusaric acid inhibit the succinoxidase and cytochrome oxidase activity of mitochondria from tomato seedlings (Paquin and Waygood, 1947).

The complex series of reactions which yield energy are probably similar in host and pathogen, and some of the reactions may be particularly susceptible to inhibition. The extent to which these particular reactions are affected determines the general rate of the series of reactions and these critical systems may be different in host and pathogen. Alternatively, the same systems in host and pathogen could be differently susceptible to the same inhibitor. Interference with the respiration of host cells may lead to the accumulation of intermediates which can be used by the pathogen or to death of the cell by interfering with its energy-yielding mechanisms.

The fact that the permeability of host cells may be affected by pH changes caused by the pathogen has already been referred to. This can also happen in other ways. Bachmann (1956) and Gäumann and Loeffler (1957) state that fusaric acid and lycomarasmin reduce the permeability of plant cells, but it is not known whether these substances act as general cytoplasmic poisons, or affect specific structures in the plasma membranes responsible for semipermeability. The latter possibility is indicated by Burchfield and Storrs (1957) who found that 2,4-dichloro-6-anilino-*s*-triazine causes the release of phosphate from fungal spores but does not impair germination. They suggested that the permeability changes depend on reaction between halogen atoms on the triazine ring and sulfhydryl and amino groups of essential enzymes associated with selective transport systems. In view of the permeability changes in cells next to infected tissue in some diseases, it is possible that some toxins of pathogens could act in similar specific ways.

5. *Other Methods of Damaging Host Tissues*

There is good evidence that vascular wilt pathogens which are confined to the xylem elements produce disease symptoms by reducing the flow of sap in the xylem. This may happen in a number of ways. Enzymes of the pathogen may act on the cell wall of the xylem elements to release high molecular weight compounds which impede the flow of the sap. Alternatively, the action of these enzymes may promote the formation of tyloses with the same effect. The pathogen may produce high molecular weight compounds which reduce the flow of sap as described by Hodgson *et al.* (1949). It has been suggested that the increased viscosity of solutions of high molecular weight substances reduces the rate of flow, but this is not always true because the effect can be obtained with dilute solutions with viscosities little more than that of water. The flow of water could be impeded by the accumulation of the high molecular weight substances at the cells surrounding the ends of the vascular elements.

The action of toxins on the respiration of host cells has already been dealt with. Substances produced by pathogens may also act in other ways because certain respiratory enzymes may require specific coenzymes for activity, and the rate of some reactions may be limited by the availability of these cofactors. It is known that microorganisms often produce certain cofactors in excess of their own requirements. If the same cofactors were limiting host respiration, an exogenous supply could lead to increased respiration of the host cells.

Recent work has shown that substances such as indoleacetic acid may influence the development of plant cells, particularly the formation of cell walls (Glasziou, 1957), by altering the degree of adsorption of pectinesterase to cell walls. This implies that pectic enzymes play a more important part in cell metabolism than has hitherto been recognized. If this were shown to be so, production of pectic enzymes in plant tissues may assume a new significance.

V. INVASION OF DIFFERENTIATED CELLS

Almost all plant pathogens first become established in tissues composed of parenchyma and many are confined to this type of tissue. A substantial proportion of young plants and a large proportion of older plants consist of cells in which the properties of the walls are profoundly modified by the deposition of materials which are mainly organic in nature. With few exceptions, these changes are followed by death of the protoplast. The more important substances deposited in the walls of differentiated cells are the mannans, galactans, arabans, and xylans (polymers of mannose, galactose, arabinose, and xylose); the polyuronide hemicelluloses which, essentially, are mixed polymers of pentoses and uronic acids; lignin, probably the most important impregnating substance, and with a complex structure still not fully determined; and suberin, characteristic of cork tissue, with a complex and incompletely known composition.

The noncellulosic polysaccharides and to a lesser extent the hemicelluloses resemble pectic materials in a general way. Although little is known about their degradation, it is probably similar to that of pectic materials. In differentiated cell walls the three types of materials are attacked only slowly, possibly because they are mixed with, and therefore protected, by other substances much less susceptible to degradation. Lignin and suberin are in this category; although they are degraded by some organisms, the process is very slow even when it is done by fungi which are adapted to this type of nutrition. Lignin and suberin are among the most resistant of all plant products to attack by plant pathogens, and cells impregnated with them form barriers which cannot be broken by most plant pathogens. Such cells are seldom penetrated mechanically possibly because their walls are thick and tough, and chemically are little affected because the pathogens do not produce the necessary enzymes, or because enzymatic degradation is so slow. When a pathogen invades tissue containing lignified or suberized elements, either it is stopped or it bypasses them in various ways (Marsh and Walker, 1932). It is probable that even after the plant is killed, most of this type of tissue is degraded in the soil, and then only very slowly.

VI. Penetration of Induced Barriers

A. *Induction of Barriers*

The barriers to infection already discussed are those present before infection. Apart from these preformed barriers, living tissue invaded by pathogens may react by producing new barriers which may be regarded as being induced by the pathogen.

A common barrier of this type is formed in certain leaf spot diseases with self-limiting lesions. Invasion is followed by death of a small group of cells around the point of entry. Cells some distance away soon form a phellogen which produces a layer of suberized cells separating the lesion from the rest of the leaf. The barrier formed presumably prevents movement of metabolic products of the pathogen into the rest of the leaf, and so restricts the spread of the pathogen. The barrier, if complete before the fungus reaches it, effectively limits the size of the lesion.

Similar barriers are often produced in leaves damaged mechanically or chemically, so that in leaf spots cork formation may be a direct consequence of the killing of cells by the pathogen.

Cork barriers develop relatively slowly and are effective only if the pathogen also grows relatively slowly because rapidly growing pathogens invade or kill the tissue before a phellogen is formed. Another possibility is that some pathogens prevent the formation of the phellogen either by secreting substances which interfere with the metabolism of the cells which otherwise would become meristematic, or by destroying the principle which diffuses from dead cells to stimulate meristen formation.

In another type of induced barrier, invasion of a cell causes it to produce substances toxic to the pathogen. Kuc *et al.* (1955) and Müller (1956) have obtained evidence for such substances in potato tubers; the toxic effects are not confined to the pathogen which causes the formation of the toxins. Similar effects have been found in a number of other host-pathogen combinations, in some of which phenols are believed to be the active substances. Whether or not substances with more specific effects are produced is still an open question. Such substances have often been the basis of attempts to explain the specialized parasitism of fungi such as the rusts. It has been supposed that penetration of a resistant cell by a particular race leads to the formation of specific "antibodies" which react with the pathogen or its toxins. The effect is specific in the sense that these hypothetical antibodies are not formed in cells invaded by a race to which it is susceptible. The parallel is, of course, with the highly specific antigen-antibody reactions which occur in animals but it is not a close one because the effect is necessarily local and generally must remain so in the absence of a circulatory system. So far there is little evidence that specific substances of this nature are produced by plant cells. Satisfactory evidence would not be easy to obtain because it would be necessary to isolate such substances from plants and to demonstrate their specific activity. If plant cells did sometimes respond in this way, virulence of a pathogen would depend on its inability to evoke a response, or to be tolerant of the substances produced.

From time to time another type of induced resistance has been described in which infection of a plant either causes it to be resistant to reinfection or causes tissues near the infected tisue to become resistant. Most examples of the former type come from field observations that successive attacks by the same pathogen become less damaging, or from experiments such as those of Tischler (1914) in which plants of *Eu-*

phorbia cyparissias cured by heat after infection by *Uromyces pisi,* were resistant to reinfection.

It is difficult to assess the importance of this and similar work because the results quoted can often be explained in other ways. In more critical work, Yarwood (1954) showed how carefully experiments of this sort must be interpreted. Circumscribed areas of bean leaves were inoculated with rust spores; some time later, adjacent areas were found to be resistant to infection by the same fungus. From this it could have been assumed that cells in these areas had become resistant, but it was shown that the effect was caused by some volatile products of the original infection which prevented the germination of the spores of the second inoculum.

On the other hand, it is well established that infection at one point can cause metabolic changes in uninfected tissue elsewhere and it is not difficult to see how resistance could be altered at the same time particularly in delicately balanced host-pathogen relationships.

B. *Development of Progressive Lesions and the Concept of Inoculum Potential*

Lesions caused by some pathogens in relatively homogeneous plant tissue grow only to a limited size, whereas lesions caused by another pathogen may continue to grow until all the available tissue is invaded. In both types the normal sequence of events can be reversed by comparatively small changes in the internal or external environment of the plant (Murant and Wood, 1957).

In a self-limiting lesion, the pathogen overcomes the barriers to infection initially present in the host tissue, but then is unable to overcome those which it produces itself, and those which it causes the host tissue to produce. The host tissue may be unsuitable for continued growth of the pathogen so that the colony formed is restricted in size as is a colony growing on an unsuitable agar medium. The pathogen by degrading the polymers present in dead cells may also cause changes in nutrient levels which would favor reproduction at the expense of vegetative growth. Host cells near invaded tissue may be stimulated either to produce substances toxic to the pathogen or to produce an impenetrable cork barrier. The effectiveness of each of these induced barriers depends upon relatively slow growth of the pathogen through the tissues.

In contrast, pathogens which cause progressive lesions may have less exacting nutrient requirements, they may be intrinsically less susceptible to toxins produced in host cells after infection, or possess mechanisms by which the activity of such substances is reduced. They may also not produce specific substances which stimulate the formation of phellogen.

It is probable that other important attributes are the ability to grow rapidly, and to secrete enzymes which disintegrate host tissue so that the effect of the products of metabolism are enhanced. In this way cumulative effects would be produced. The defenses of the host are overwhelmed and barriers effective in the self-limiting lesions are not formed. Under such conditions, the only factor limiting colonization is the suitability of the host tissue for growth and enzyme secretion. If this be so, the formation of a progressive lesion depends largely on the ability of the pathogen to produce pectic and other enzymes which disintegrate plant tissues, the early stages of this process being particularly important. Comparable effects have been described in animal pathology where it is thought that the hyaluronidase secreted by some pathogenic bacteria may increase their "invasiveness," by degrading the materials which unite the cells of a tissue.

The concept of "inoculum potential" (Horsfall, 1932; Garrett, 1956) will now be dealt with briefly because some interpretations of this expression are related to the problems associated with the development of progressive lesions.

"Inoculum" is still used in a variety of ways but, generally, it has come to mean a part of a microorganism which will cause disease when associated with another organism under the right conditions. Thus, in a chamber containing spores of *Venturia inaequalis* and apple and grass leaves, none of the conidia would be inocula for the grass, but all viable conidia would be inocula for the apple leaves although some might not be able to cause infection under the prevailing conditions. Here, the population of viable conidia is the inoculum potential; it could also be called the "potential inoculum," or, in the sense given above, simply the "inoculum."

In this example the inoculum potential may be measured more or less precisely by counting the number of viable conidia in the chamber, but in other host-pathogen combinations measurement of the inoculum is much more difficult particularly when the pathogen is soil-borne. In such cases, the inoculum potential is estimated by placing susceptible plants in the environment and measuring the proportion which becomes diseased. The figures obtained will depend on the condition of the host and on the environment and will give a true picture of the inoculum potential only when conditions are optimal for the development of disease.

Inoculum potential used in the above way is the amount of infective material present in a given environment.

Garrett (1956) has used "inoculum potential" in a different sense defining it as "the energy of growth of a fungal parasite available for infection of a host at the surface of the host organ to be infected."

(Presumably, this definition can be considered to apply also to bacteria and other organisms infecting plants.) In this definition, "inoculum potential" is the potential of an inoculum for infection. It has the advantage of implying that different inocula of a pathogen have different potentialities for infection, but has the disadvantage of relating this potential to "energy of growth" so that the definition hinges on what is meant by growth. By this is commonly meant the ability of an organism to increase in size, accumulate more protoplasm, and so on. But an ability to grow may not be related to an ability to infect and later to cause disease. Infectivity may depend primarily upon the production of a particular enzyme or toxin, and this may happen at the expense of materials which otherwise would be used for growth. When used in this way, the potential of an inoculum becomes more or less synonymous with its virulence, that is, with its ability to become established in a host, and it is in this sense particularly that the concept is relevant to the subject of this chapter.

In animals it is well known that in many diseases the inoculum must reach a certain size before the pathogen becomes established, and that virulence can be altered by factors such as those responsible for the transformation of rough, avirulent types of pneumococcus into smooth, virulent types. At present there is little precise information about the operation of similar factors in plant diseases although some have been recognized in certain root diseases where the inoculum is generally large, consisting of a mass of hyphae attached to a food base. The ability of an inoculum of this sort to invade and colonize fresh tissue may depend on the secretion of substances in sufficient quantity to destroy the barriers present in the tissue, and this in turn may depend on the size of the inoculum, and sometimes, at least, on the size and nature of the food base. Once the pathogen has become established, the functions of the food base are taken over by the freshly colonized tissue. In diseases of shoots, the inocula are almost always in the form of spores, the internal reserves of which correspond to the food bases of the root-infecting fungi. A pathogen may become established in tissues of a shoot only if substances which destroy the barriers to infection accumulate in sufficient quantity at the site of infection. This could result from the combined action of a number of weakly virulent inocula, each producing a small quantity of the active substances, or because a single virulent inoculum was able to secrete relatively large quantities of the same substances. Unfortunately, little is known qualitatively or quantitatively about this aspect of infection and about the different potentialities of inocula to infect plants. It calls for a comparative study of the infection of standard host plants under controlled conditions by different numbers of the same types of inocula, and by the same number of inocula produced under different conditions, or subjected to different treatments after their formation.

VII. CONCLUSIONS

For the purposes of this final discussion the resistance of a plant to invasion and colonization by a microorganism can be considered as the sum of the factors which make the tissues of the plant, whether living or dead, unsuitable substrates for the continuous growth of the microorganism. With obligate parasites it is generally thought that resistance takes the form of a hypersensitivity of the cells to the parasite so that they die soon after they are invaded, and, therefore, become unsuitable substrates because obligate parasites can obtain nutrients for growth only from suitable living cells. It is assumed that the cells are killed by the metabolic products of the pathogen, either because they are particularly sensitive to what might be considered the products of a normal metabolism of the pathogen, or because the host cell causes the metabolism of the pathogen to become abnormal, the cells then being killed by the by-products of a deranged metabolism. There is also the possibility that by its growth the pathogen depletes the host cell of essential nutrients, and by continuing to do so kills the cell in this way.

The ability of a microorganism to parasitize a tissue will, therefore, depend upon its capacity to use the nutrients which the cell does or can provide, and at the same time not secrete substances toxic to the host cells. It may also depend upon an ability to modify the metabolism of the host cell so that the cell provides certain essential nutrients at levels which will satisfy its own requirements as well as those of the pathogen.

Most nonobligate parasites kill the cells of invaded tissues and, for the most part, derive nutrients from dead cells. Generally, the cells are moribund for some time before they are killed so that the substrate for the pathogen is one in which there may be many substances not present in healthy cells, substances formed while the cells are functioning abnormally under the influence of the metabolic products of the growth of the pathogen, and those formed in the series of degradative changes which follow death of the cells. In resistant plants, the substrate provided by the dead cells may be unsuitable for the growth of a pathogen simply because it contains, from the beginning, one or more toxic substances. It is remarkable how seldom this has been demonstrated. More often it is likely that dead tissue becomes unsuitable for continued vegetative growth and for the production of substances which kill host cells, by the activity of the pathogen itself. If it can be shown that the dead tissues of a resistant plant possess none of the above properties, it must be assumed that the pathogen induces changes in living cells adjacent to the area already invaded, and that these changes make the cells resistant while they are still living, or make them unsuitable substrates after they have been killed. A nonobligate parasite will colonize a tissue if it is able to kill cells after a limited growth in living cells or in tissues killed beforehand, and able to use the dead cells for growth and production of substances by which it continues to kill the cells. At the same time it must not produce substances which induce changes in living cells of the host which make them unsuitable substrates. If these conditions be satisfied, the pathogen will continue to invade the tissue and form a progressive lesion. Such tissue so invaded is then regarded as susceptible.

Our understanding of the complementary problem of resistance of the host, and virulence of the pathogen will, therefore, depend on knowing more about the reactions of the host cells to the metabolic products of fungi and bacteria, about the physiology of the pathogen with complex organic substances as substrates, and about the ways in which nutrients affect the production of substances by which pathogens produce their effects upon host plants.

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CHAPTER 8

Interaction of Pathogen, Soil, Other Microorganisms in the Soil, and Host

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I. INTRODUCTION

The interaction of soil microfloras on plant growth exhibits considerable plasticity. For instance, certain microorganisms, particularly bacteria, enter into a commensalism with roots and help certain leguminous plants in acquiring the all important nitrogen for their metabolic needs. The peculiar symbiotic relationship of the endotrophic and ectotrophic mycorrhizal fungi with root systems of certain forest trees is another facet of the problem. Heterotrophy among mycorrhizal fungi became evident with the recent discovery of the B vitamins. Radiophosphorus was shown to be absorbed by endotrophic mycorrhizal fungi and transferred to the host tissue.

Nevertheless, there are other relationships between plants and microorganisms of the soil that are not looked upon with favor since their destruction of crops can deprive man of one of his primary needs. Microorganisms may compete with plant roots for essential energy substrates for development of enzyme systems and primary cell functions.

It therefore becomes imperative to study interactions between pathogens and other microflora of the soil and also between pathogens and the host root systems. Around this has grown the new concept of rhizosphere microfloras and their repercussions on host-pathogen relationships. This shift in emphasis is fortified by the discovery that many amino acids, sugars, organic, and inorganic substances are exuded from living root systems and that antibiotics and other metabolites are constantly being produced *in situ* in soils. The uptake of these complex microbial metabolic products is no longer a matter for speculation and there seems every reason to believe that these interfere *in vivo* with ionic balance and lead to consequent enzyme dysfunction. Clearly, therefore, in the study of soil-borne root infections we have to consider the varied and complex interactions between pathogen, soil, and host.

II. Pathogens in Soil

Soil microbiology as a science has come into its own and shown that there is a dense population of varied microbes in the soil. This microbial population includes several hundreds of forms of fungi, many bacteria as well as actinomycetes. It includes simple saprophytes and plant pathogens. The pathogenic forms are capable of infecting roots of suitable hosts and cause disease. The very stable tobacco mosaic virus and certain other viruses such as wheat mosaic, lettuce big vein, and tobacco necrosis viruses, which lose their infectivity much more rapidly, are soil-borne (see Bawden, 1950).

Soil-borne plant pathogens differ in the type of disease they produce and these root diseases may be classified on the basis of the nature of the tissues attacked and the effects produced.

Parenchyma diseases are those in which the parenchyma alone may be the center of attack and may be disintegrated. These diseases may be caused either by soil bacteria or fungi. Soft rot of carrots, for example, is caused by *Bacterium carotovorum* and the pathological effects produced result from the separation of the individual cells of the parenchyma due to enzyme action whereby the middle lamella between adjacent cells is dissolved. On the other hand, several species of fungi belonging to the genera *Pythium, Phytophthora, Rhizoctonia,* etc. cause root rots, foot rots, stem rots, and damping-off of seedlings. In all these cases, rotting may be due to death of cells or to the dissolution of the middle lamellae. The early work of de Bary and of Marshall Ward indicated that, following penetration of host tissue, *Sclerotinia* and

Botrytis secreted enzymes which diffused in advance of the pathogen and led to the breakdown of parts of the cell membrane. The chief enzymes partaking in the macerating effect of many fungi and bacteria on plant tissues are discussed in Chapter 5, Volume I and Chapter 7, Volume II, and their role in root diseases will be discussed in detail later.

Vascular diseases are those in which the vascular tissues, particularly the xylem, may be invaded and blocked resulting in wilting. These may also be caused by bacteria or fungi. As examples of bacterial vascular wilt may be cited the gumming disease of sugar cane due to *Xanthomonas vasculorum* and bacterial wilt of maize due to *Xanthomonas stewartii.* Vascular wilts due to fungi are many and are caused by species of *Fusarium, Verticillium, Valsa,* etc. The symptoms usually include various degrees of chlorosis, vascular discoloration, stunting, and wilt. Infection takes place through root systems and the pathogens are normally confined to the vascular elements except during the final stages of the disease when the plant tissues are dead and the pathogens grow out from the vascular elements. Cotton wilt due to *Fusarium vasinfectum* and tomato wilt due to *F. oxysporum* f. *lycopersici* are good examples.

There are then what may be called *systemic diseases* in which both parenchyma and vascular tissues may be invaded. For instance, in the case of brown rot of solanaceous plants due to *Pseudomonas solanacearum*, the symptoms are those of a general wilt followed by collapse. In most affected plants, a brown stain is invariably formed in the xylem. If infected stems are cut across, the bacteria ooze out in drops. In contrast to the vascular wilts, the pith and the cortex are invaded later. Distinct cavities filled with bacteria are formed in the pith region and the cortex may undergo disintegration.

There are also many root infections which lead to *hyperplastic diseases*, which are discussed in Chapter 6, Volume I. Clubroot of crucifers due to *Plasmodiophora brassicae*, potato wart due to *Synchytrium endobioticum*, and crown wart of alfalfa due to *Physoderma alfalfae* are well known examples of hyperplastic diseases of fungal origin. Hairy root and crown gall of plants, on the other hand, are induced by bacteria infecting root systems. What is noteworthy about these galls is the capacity of unlimited and unrestrained proliferation acquired by some of their cells in the absence of the crown gall bacterium.

III. PATHOGENS AND ROOT INFECTION

A. *Facultative and Obligate Parasites—Host Range*

In the etiology of root diseases, infection of root systems takes place either by an obligate parasite or a facultative one, or sometimes even by quite a different agent such as a virus. Problems relating to obligate and facultative parasites have been admirably discussed by Brooks (1948) and Brown (1948) and it would be sufficient here to consider the question of obligate and facultative parasitism insofar as it is peculiar to root-infecting pathogens. Garrett (1956) has built up an evolutionary sequence beginning with the obligate soil saprophytes lacking the ability to parasitize living plants and ending with what he terms "ecologically obligate parasites," whose relationship with the hosts is one of symbiosis. The intermediate links in this evolutionary chain are (1) the primitive parasites that destroy seedlings and juvenile tissues and can live saprophytically; (2) the less primitive parasites which rapidly destroy plant tissues and are less restricted by the host than (1); (3) the specialized parasites that cause less disorganization of the host tissues and do not exhibit saprophytic activity other than passive survival in tissues invaded as parasites.

Although Garrett's classification refers to root-infecting fungi, it should be applicable to other root-infecting pathogens also. However, in our view, this evolutionary sequence does not necessarily imply an inverse relationship between parasitic specialization and saprophytic ability such as has been underlined by Garrett throughout his treatise on the biology of these pathogens. A facultative parasite, for instance, in having acquired the ability to parasitize a host, certainly need not be considered to have lost its saprophytic ability even in a small measure. In other words, it is not less of a saprophyte than the so-called obligate saprophyte because it has acquired the ability to parasitize a host plant.

That this is so becomes obvious from a comparative analysis of the competitive saprophytic ability and parasitic specialization seen among root-infecting fungi. For instance, comparison may be made between *Fusarium vasinfectum*, the cotton wilt pathogen, and *Sclerotium cepivorum* which causes white rot of onions and related hosts. The former causes a typical vascular wilt and, in its relationship with the host, hardly causes any decay or necrosis, but normally invades the xylem elements whence it produces metabolites (vivotoxins) whose activity on host tissues leads to wilting. *Sclerotium cepivorum*, on the other hand, causes a rot of the host tissues, presumably by enzymatic action. Insofar as *Fusarium vasinfectum* causes little necrosis of host tissues and tends toward a somewhat balanced relationship with its host, it may be considered more specialized than *Sclerotium cepivorum*. When, however, the competitive saprophytic ability of these two pathogens is compared, it is found that they are closely similar. That *Fusarium vasinfectum* makes little free mycelial growth in unsterilized soils, but is capable of long periods of survival therein as a colonizer on dead plant remains, is now an established fact (Subramanian, 1950). *Sclerotium cepivorum* behaves in exactly the same way, as shown by the recent work of Scott (1956a, b). Thus, *Fusarium vasinfectum*, notwithstanding its parasitic specialization, possesses as much competitive saprophytic ability as that shown by *Sclerotium cepivorum* and, indeed, both may be grouped as soil inhabitants, as defined by Garrett (1956). On the other hand, *Fusarium udum*, which causes a vascular wilt of *Cajanus cajan* similar to cotton wilt, appears to possess much less competitive saprophytic ability than *Fusarium vasinfectum*, although the parasitism of these two pathogens is practically of the same order of specialization. Indeed, in the case of *F. udum* it has been shown that it can persist in soils only as a colonizer on host tissues which it originally invaded as a parasite (see Subramanian, 1954), a behavior strikingly reminiscent of that of *Ophiobolus graminis* (see Garrett, 1956). These examples clearly show that little support can be accorded to the view that specialization of parasitism brings along with it loss in competitive saprophytic ability. While this is so in some cases, it certainly cannot be a general rule and cannot, therefore, form the backbone of any logical arrangement of behavior patterns of these organisms.

The concept of "ecologically obligate parasites" (Garrett, 1956) proposed to take in the mycorrhizal fungi, again suggests that the mycorrhizal habit is the end product of specialized parasitism. This may be so in some cases but, as pointed out by Bawden (1957), "there seems no need to assume that such conditions only arise from a long period of evolution, or that parasites must always evolve from virulence through avirulence to symbiosis."

The subject of host range necessarily requires treatment in any consideration of parasitic behavior of root-infecting pathogens. Here again an extraordinary range in variability is met with, with many pathogens showing preferential pathogenicity on a few host species and others being omnivorous. Between these two extreme types intermediate forms also occur. Parasitic specialization leading to balanced or obligate parasitism need not carry with it restrictions in the choice of hosts, although this does occur in some cases. Conversely, some facultative parasites, although undoubtedly less specialized than the balanced ones, may exhibit a high degree of specialization in host choice. Consider, for instance, *Plasmodiophora brassicae* which causes clubroot of crucifers. For a long time, this obligate parasite was considered to be capable of infecting only cabbage and other crucifers, but the recent work of Webb and of Macfarlane (see Macfarlane, 1952) has removed this misconception. From the work of these investigators it is clear that this fungus can infect root systems of noncruciferous plants such as *Tropaeo-*

lum majus, *Reseda odorata*, *Papaver rhoeas*, *Agrostis alba* var. *stolonifera*, *Dactylis glomerata*, and *Lolium perenne*. In none of these, however, is the fungus known to produce clubroot symptoms following infection. Root hair infection of seedlings of *Matthiola incana*, *M. bicornis*, and of *Lepidium sativum* has also been observed and in *L. sativum*, infection was followed by clubroot symptoms. Further, among the Cruciferae, the pathogen has a wide host range. Similarly, *Synchytrium endobioticum* is another obligate parasite which infects, besides potato and tomato, *Solanum nigrum*, *S. dulcamara*, and other species of *Solanum*, *Hyoscyamus niger* and *Nicandra physaloides*. The host spectrum of this fungus is thus limited to the Solanaceae. *Spongospora subterranea*, which causes powdery scab of potatoes, likewise infects a few other hosts belonging to the Solanaceae. A further restriction in host range is met with in the case of another obligate root parasite, *Physoderma alfalfae*, which infects only common alfalfa, *Medicago sativa* and *M. falcata*. It would appear that none of the root-infecting obligate parasites shows preference to less than two host species.

The greater number of root-infecting pathogens, no doubt, are of the facultative type and among these also we do find a narrow or a wide host range. Species of the genera *Pythium*, *Sclerotinia*, *Rhizoctonia*, *Armillaria*, *Fomes*, *Ganoderma*, *Fusarium*, and *Verticillium*, to cite only a few examples, are omnivorous and can infect an appallingly wide range of hosts which may be closely related or not related at all. Other facultative parasites appear to have a host spectrum limited to related plants belonging to a single family or sometimes to a single genus. Thus, *Sclerotium cepivorum*, *Botrytis allii*, and *Colletotrichum circinans* can infect only species of the genus *Allium*, and *Fusarium oxysporum* f. *lycopersici* attacks only two species of *Lycopersicon*. Examples may also be cited here from among soil-borne bacterial pathogens. *Corynebacterium fascians*, which causes leafy gall and fasciation in chrysanthemum, dahlias, etc., is apparently specialized in its parasitism since it causes hyperplastic disease, but is able to infect plants belonging to different families. *Pseudomonas solanacearum* causes brown rot of several Solanaceae, and *Bacterium tumefaciens* infects a large number of related and unrelated host species. On the other hand, *Xanthomonas hyacinthi* infects only a single species, *Hyacinthus orientalis*, and *Xanthomonas vasculorum* infects only sugar cane.

The examples just cited include root-infecting pathogens of varying degrees of specialization in regard to the types of disease caused. Progressive limitation in host range does not necessarily accompany progressive specialization in parasitism of root-infecting pathogens. These two are obviously independent tendencies which have found expression to varying, but not necessarily equal, degrees in different root parasites.

B. *Infection: Prepenetration and Penetration Phases—Physical, Chemical, and Physiological Aspects*

Root infection is an essential condition for root disease. The process of infection in root disease has been studied by a number of workers and the principles underlying root infection are in general much the same as those that hold for aerial infections. Spores of pathogenic fungi or inocula of other disease causing agents occur in soils, often in great abundance, and in the case of sporulating forms there are obviously a number of factors which would affect their germination. Indeed, in the case of all pathogens, infection depends on various factors: (a) viability; (b) conditions suitable for germination and penetration. In any given soil, spore germination depends largely on a suitable combination of moisture content, temperature, oxygen supply, pH and, in some cases, the presence of suitable nutrilites.

Many years ago, Brown (1948) showed that there is a certain amount of "exosmosis" of materials from host tissues, leading to a chemotropic stimulus for spore germination and infection. This postulate is particularly true of soil-borne pathogens since it is known that root exudates from living plant roots may influence spore germination and root infection considerably. For instance, Noble (1924) showed that germination of spores of *Urocystis tritici* was stimulated by roots of nonsusceptible plants, e.g., pea, bean, and rye, and also by traces of benzaldehyde, salicylaldehyde, acetone, and butyric acid. Macfarlane's work (1952) indicates that similar stimulation of resting spore germination in *Plasmodiophora brassicae* is brought about by several cruciferous and noncruciferous species resistant to clubroot. In the same way, *Datura stramonium* has been reported to stimulate germination of spore balls of *Spongospora subterranea*, without the subsequent development of the disease in the root system (White, 1954). The potato plant is known to secrete into the soil substances tending to break the dormancy of spores of the potato wart fungus, *Synchytrium endobioticum*. Varieties of flax resistant to wilt caused by *Fusarium oxysporum* f. *lini* are known to excrete hydrocyanic acid through their root systems and this was shown to depress the growth of the pathogenic *Fusarium* and also of *Helminthosporium*. Further, hydrocyanic acid was also shown to encourage the growth of saprophytic fungi such as *Trichoderma viride* which are antagonistic to plant pathogenic forms (Timonin, 1941). Root excretions of susceptible flax varieties do not appear to contain appreciable amounts of hydrocyanic acid. In the same way, Buxton (1957a, b) found that exudates from the roots of pea varieties stimulate the germination of spores of races of *Fusarium oxysporum* f. *pisi* to which the varieties are susceptible and inhibit that of races to which they are resistant. He also found that, when a variety resistant to the race present in the soil was grown, wilt incidence of a susceptible variety grown in that soil subsequently decreased.

These observations are of great significance in understanding the biology of root infections since they indicate that spore germination around the rhizosphere and subsequent infection would be greatly influenced by exudations from susceptible and nonsusceptible root systems. Where stimulation of spore germination is achieved with the aid of nonsusceptible plants, possibilities of reducing the effective survival of spores in soil and bringing down the inoculum potential to an innocuous level have been envisaged. However, little is known about the stability of these exudates in soils and it is probably not likely that they remain effective to a sufficient extent in space and time to be active in breaking spore dormancy.

The infection process itself deserves to be discussed here in some detail since we do find among the root-infecting fungi distinctly different patterns of behavior. De Bary's hypothesis of "killing in advance of penetration" proposed to explain infection by facultative parasites was challenged and disproved by Brown (1948) who worked with *Botrytis cinerea* and presented evidence showing that the host cells are not killed by this facultative parasite until after it penetrates the cuticle of the host. According to Brown, direct penetration through cutinized walls is believed to be entirely by mechanical pressure, since no cutin-dissolving enzyme has been demonstrated in fungi. The root-infecting fungi normally encounter a host substratum devoid of cutin. Penetration of such noncutinized walls may be either by mechanical pressure or by the dissolving action of enzymes secreted by the fungus, or both. Many pathogens enter the host at the points of emergence of lateral rootlets and several infect through wounds. The so-called "wound parasites" are those which cannot infect by direct penetration but only through wounds. Such wounds may be the result of root injury due to various causes, including injuries due to animals.

Finally, mention must be made of root infection by plant viruses (Bawden, 1950). There is evidence that such infection does occur, notwithstanding the conflicting reports published by different workers. For tobacco mosaic virus it has been claimed repeatedly that the soil is a common source of primary infection; on the other hand, it has also been shown that root infection does not take place commonly even if the roots are deliberately wounded. In the case of tobacco necrosis viruses, the roots get easily infected, but there is little movement of the viruses from the roots to the tops and the tops show no symptoms. This is surprising in view of the fact that when the leaves are inoculated with the viruses, they spread to all plant parts including the roots. That this reluctance to invade the tops from the roots is not common to all viruses is shown by the work of Roberts (1946) who found that potato virus X can spread from infected to healthy plants which are in contact only below ground, and tomato plants infected through their roots showed leaf symptoms.

Granting that parasitism and specialization are later products of an evolutionary tendency, we have then among the root-infecting pathogens infection behavior patterns from the simple to the highly specialized. The forms that "kill in advance of penetration" should possibly find a place low in this scale of evolution, followed by the so-called "wound parasites." The forms that show the ability to penetrate mechanically the host surface are evidently more specialized. The peak of this tendency is found in some pathogens such as *Ophiobolus graminis* causing take-all of wheat (Garrett, 1956) and *Phialophora radicicola* attacking maize roots (McKeen, 1952) where the fungi spread extensively along the living root systems of the hosts mainly by brown runner hyphae and produce, in addition, fine infection hyphae which penetrate the host tissue at many points. Finally, it is a point of more than ordinary interest that most of the obligate root parasites are capable of direct penetration of at least younger root tissue.

C. *Factors Concerned in Pathogenicity*

1. *The Pathogen: Inoculum Potential; Physiological Requisites for Pathogenicity and Disease Development—Enzymes, Vivotoxins, Antibiotics, etc.*

Since infection is the first step in pathogenesis, presence of the pathogen is also implied as a prerequisite. Usually, mere presence of the pathogen alone is also insufficient for successful infection and pathogenicity; inoculum of the pathogen will have to be present in quantity. Inoculum potential, then, is the effective concentration of inoculum per unit area of root surface needed for pathogenicity (Garrett, 1956). The term thus connotes not only presence but also effectiveness of the inoculum. Both the concentration and effectiveness of inocula in soils will depend on the many factors, which are fully discussed in Chapter 2 of Volume III. Depending on the conditions under which the host and the pathogen interact, and also the nature of the host and the pathogen, a minimum concentration of infecting units may be needed for successful infection. Following Gäumann (1950), this may be termed the numerical threshold of infection. Where, as in susceptible hosts and virulent pathogens, the chances of infection are high, the numerical threshold is low. In the case of root diseases in general, a single spore may be ineffective. In the case of potato wart, for example, 200 resting sporangia of *Synchytrium endobioticum* per gram of soil are necessary to cause disease. Similarly, in the case of snow mold of wheat due to *Fusarium nivale*, at least 10,000 conidia per milliliter of inoculum are needed; in tomato wilt due to *F. oxysporum* f. *lycopersici*, even with a virulent strain, 700,000 conidia per milliliter of inoculum are essential for pathogenicity. The further need of a food base providing nutrients is seen in many root-infecting pathogens such as *Fomes lignosus*, *Armillaria mellea*, *Xylaria mali*, *Phymatotrichum omnivorum*, and *Rhizoctonia crocorum*.

Many other factors influence pathogenicity, among which are enzymes, vivotoxins, and antibiotics. The essentiality of enzymes for living systems is generally appreciated. It did not, however, fall to the lot of plant pathologists to unravel the mysteries of enzyme chemistry of phytopathogenic fungi and bacteria until the early twenties when Brown (1915) initiated his studies on enzyme systems of fungal facultative parasites and, indeed, he has recently reviewed the entire field in a masterly fashion (Brown, 1955).

From the plant pathological point of view, pectolytic enzymes that are known to attack cell wall constituents and their production *in vitro*, and the ultimate correlation of this knowledge of *in vivo* functional mechanisms have found much favor and many active preparations from fungi, such as *Botrytis cinerea*, *Pythium* spp., and many soft rotting bacteria have been made. There is, however, no parallelism possible between the pathogenic potentialities of the organism and its *in vitro* enzyme production. The role of pectic enzymes in tissue disintegration is discussed in Chapter 5 of Volume I, and Chapter 7 of Volume II, and need not be elaborated here.

Wherever there is evidence of enzymatic action on the cell wall it is generally conceded that there would appear no need to postulate the presence of a killing agent. It is true that there is evidence for a number of microbial metabolites injuring host protoplast, but not in all cases have there been justification and experimental proof for their production *in vivo* in sufficient concentration to have caused the injury. There seems little doubt that substances of large molecular sizes can be dislodged or liberated by enzymatic action on cell pectic substrates and these can create a situation in vessels and possibly in the pores of pit membranes resulting in clogging and consequent internal resistance to water conduction.

Much applied work on wilt toxins (See Chapter 9, Volume II) and application of this fundamental knowledge of pectolytic enzymes has been reported in recent years. Reviews on the subject by Dimond (1955) and Walker and Stahmann (1955) have admirably summarized these facts.

It is this newer concept born of fundamental studies on pectolytic enzymes of the cell that has to be considered next under the head vivotoxin. In other words, the sequence of enzyme action, and vivotoxins have now to be discussed *ad lib* in the succeeding paragraphs commensurate with the progress made in their study. This new term "vivotoxin" has been defined as a substance produced by the pathogen or its host, or both, which operates in the causation of disease but is not in itself the primary agent (Dimond and Waggoner, 1953a). Lycomarasmin produced *in vitro* by *Fusarium oxysporum* f. *lycopersici* does not seem to answer the test and is probably not a vivotoxin, but fusaric acid (produced *in vitro* by a number of fusaria: *F. vasinfectum, F. heterosporum, F. oxysporum* f. *lycopersici, F. orthoceras, Gibberella fujikuroi*) is now regarded as a certainty in opening the new list of vivotoxins (Lakshminarayanan and Subramanian, 1955) along with ethylene produced in *Fusarium* cultures (Dimond and Waggoner, 1953b). Relatively little, therefore, is known of this group of toxins and it would be necessary to analyze critically newer toxins of microbial origin that affect plants and assign them by grouping together metabolites with specificity of action in the causation of disease syndrome.

Antibiotics of interest to plant growth and disease production have been in the limelight for well over a decade and this subject has been reviewed more frequently than any other branch of plant pathology (Brian, 1949, 1957; Gäumann, 1954, 1957). Much work has been done on the stability of antibiotics in soils when added from external sources, and positive proof of their remaining potent for long periods under varying microbial antagonism has been obtained. Likewise, the production of antibiotics in soils *in situ* by common soil organisms not only has been proved to occur in many types of soils but it is now considered to be of fairly widespread occurrence. Furthermore, the movement of quite a number of antibiotics from soils into plants, through their roots into their leaves, with considerable rapidity, depending on the metabolic state of the plant and its environment, is now an established fact.

The role of vivotoxins in plant disease is a related subject, discussed in Chapter 9 of Volume II. Fusaric acid, a pyridine-carboxylic acid, is an example of a vivotoxin and, fusaric acid, like lycomarasmin, has metal binding properties (Lakshminarayanan and Subramanian, 1955). Much work done with *F. vasinfectum* and neat fusaric acid on the cotton plant has recently been summarized (Sadasivan, 1958). Some of the highlights may be mentioned here. The addition of zinc to *in vitro* cultures of *F. vasinfectum* increases the output of fusaric acid, the optimum being 0.24 mg. per liter and levels higher than this inhibit the production of this toxin (Kalyanasundaram and Saraswathi-Devi, 1955). Possibly, nonprotein source of nitrogen in the host is more conducive to fusaric acid output, and diploid susceptible varieties of cotton have less protein and more of nonprotein nitrogen. Cystine occurring in higher quantities in the resistant tetraploid cotton variety tested appears to be a limiting factor to wilt and presumably to fusaric acid output *in vivo* (Lakshminarayanan, 1955). Curiously enough, soil amendments with zinc—and growing susceptible plants in them—result in the liberation of cystine even in these plants which appears to confer resistance to *F. vasinfectum* in infested soils, and the susceptible variety virtually behaves like the resistant one (Subramanian, 1956). Growing susceptible diploid cotton plants in inoculated soils at temperatures of 32.5°, 35.0°, and 37.5° C. resulted in an apparent recovery or masking of symptoms at 37.5°, whereas at the two lower temperatures wilt symptoms were prominent. Such recovered plants, however, showed higher quantities of fusaric acid and cystine than low temperature ones, indicating that this excess cystine chelated with some heavy metal(s) *in vivo*, thus rendering them unavailable for fusaric acid potentiation (Kalyanasundaram and Subba-Rao, 1957). Spectrochemical analysis of the ashes of susceptible diploid and resistant tetraploid cottons, healthy and infected, showed depletion of the key element potassium in tissues of infected susceptible plants, whereas calcium, magnesium, and other elements were on the increase (Sadasivan and Saraswathi-Devi, 1957). However, the over-all picture was one of increase in metallic accumulation as shown by greater conductivity in the diseased susceptible plants (Gnanam, 1956). Further, there was a basic difference in the pattern of ionic absorption between the diploid and the tetraploid plants, the former taking up much higher quantities of cations than the latter. It appears that further studies on the plasma membranes and the gene controlled mechanisms of these genetic materials and its consequent repercussions on *in vivo* chelations with vivotoxins may add considerably to our knowledge of the behavior pattern of host plants under toxemia.

Pathogenesis is a connected sequence of events involving extraneous sources of energy substrates in the soil such as cellulose, essential minerals, vitamins, sugars, protein breakdown products including a multiplicity of amino acids, and, in fact, complex fractions such as root exudations and products of microbial synthesis such as the enzymes, vivotoxins, and antibiotics. These should be assembled before us if we are to understand every stage leading to reversible and irreversible *in vivo* changes in plants.

Recent investigations by Paquin and Waygood (1957) seem to lead one on to a new approach. They have indicated the presence of an active cyclophorase system capable of oxidizing the principal acids of the Krebs cycle in mitochondria from hypocotyls and cotyledons of tomato seedlings. Lycomarasmin and fusaric acid, produced by *Fusarium oxysporum* f. *lycopersici*, inhibit the succinoxidase and cytochrome oxidase activity of the mitochondria at a concentration of 10^{-2} *M*. The inhibition was overcome by the addition of catalytic amounts of cytochrome c. No effect was observed on succinic dehydrogenase. The toxins seem to inhibit enzyme activity by affecting the integrity of the mitochondria allowing diffusion of cytochrome c from active sites. These authors support the idea that lycomarasmin and fusaric acid could possibly affect host metabolism by removing cytochrome c from the active sites, thus causing disturbances in linked-enzyme systems rather than by destroying the semipermeability of the plasma membrane. They also categorically state that lycomarasmin and fusaric acid are not involved in the wilting of tomatoes because concentrations of toxins required for the complete inhibition of the succinoxidase activity of tomato mitochondria appear to be too high for these toxins to be specific inhibitors and, further, the lycomarasmin inhibition of the succinoxidase activity was not increased by the presence of free iron ($FeCl_3$). They, however, admit that the observation of the presence of free iron leading to potentiation of lycomarasmin is a valid one. In other words, iron chelation *in vivo*, already referred to, is not ruled out.

The enzyme story is a sound enough argument for the initiation of tissue disintegration and especially middle lamellae of cells but may not itself be adequate to explain the whole sequence of events. The rapidity of movement of the vivotoxins must be studied more vigorously. If cytochrome c disturbance is not to be considered to explain fully the alleged loss of semipermeability of the plasma membrane as indicated by ionic derangement, further experimental work has to be undertaken to find out alternative explanations for this rapid ionic imbalance. A wider range of genetic plant material should be used inasmuch as the pattern of movement of ions appears to be very different in diploid and tetraploid cotton plants (Sadasivan and Saraswathi-Devi, 1957). One shrinks from suggesting the inclusion of polyploids, allotetraploids, and other genetic materials.

2. *The Host: Resistance or Susceptibility*

That pathogenicity will also depend on the innate nature of the host is too well-known to require any detailed treatment here. Varietal resistance or susceptibility to root disease caused by both obligate and facultative parasites is quite common. For example, varieties of potato resistant and susceptible to wart have been known for a long time. Invasion of all varieties does occur, but in those which are highly resistant, the pathogen fails to induce any symptoms and no resting spores or prosori are produced (Walker, 1957). In fact, a very wide variability in response to infection occurs, some varieties producing no warts or only very tiny ones, and others inducing the formation of conspicuous and large warts. In the case of cabbage yellows due to *Fusarium oxysporum* f. *conglutinans*, two types of resistance are known, one of which (type A) is inherited qualitatively and the other (type B) quantitatively.

Susceptibility or resistance may often, but not always, be modified by the soil environment and by host nutrition. For instance, resistance of the tomato variety Marglobe to fusariose wilt is markedly influenced by the type and concentration of host nutrition, but the high resistance of the red-currant tomato is not affected. Similarly, with cabbage yellows, resistance of type A plants is not affected by host nutrition, whereas in both the type B resistant plants and susceptible plants disease percentage is inversely proportional to concentration of nutrients and to level of potassium supplied.

3. *The Soil: Physical, Chemical, and Biotic Factors; Cultural Practices*

That the environment has a marked influence on infection and pathogenicity is well known. In the case of root diseases, the soil environment is of particular importance. This environment includes not only the rhizosphere of the host plant but also the soil away from the rhizosphere.

The soil environment reflects the combined effects of a number of factors: physical factors such as soil type and texture, moisture, aeration, pH, and temperature; chemical factors which include the mineral status (nitrogen, phosphorus, potassium, and heavy metals) and organic matter of the soil; and biotic factors. Besides these, the soil environment is usually modified also by cultural practices. Recent research shows that the soil environment does not usually permit an easy analysis of its effects. In the case of fusariose wilt of pea, for instance, 28° C. is the optimum temperature for the growth of the pathogen, but the temperature-disease curve rises from a minimum at 15° to an optimum at 21° and then falls again to another minimum a little above 30°. In sand culture, however, the temperature-disease curve shows a peak at 28°, conforming largely to the temperature-pathogen curve. These observations are suggestive of the operation of the microbial factor as a modifying influence on pathogenicity in soils alone but not in sand culture (see Walker, 1957). Similarly, the temperature-disease curve may also be modified by the nature of the host. This effect is well seen in the case of *Gibberella zeae* causing seedling blight of wheat and of corn. The temperature-growth curve of this pathogen is similar to that of the pea wilt *Fusarium* and appears not to be directly related to the temperature-disease curve. This curve shows an optimum of 20° C. or above for wheat, a low temperature plant, and an optimum below 20° for corn, a high temperature plant. Thus, with both hosts, the fungus is least pathogenic at the optimum temperature for the growth of the host.

The interaction between the various physical, chemical, and biotic factors of the soil in influencing root diseases is best illustrated by data obtained on soil microfloras and their effects on soil-borne diseases. Cotton wilt, due to *Fusarium vasinfectum*, has been intensively studied from this angle and a summary of the highlights of the results obtained (see Sadasivan and Subramanian, 1954; Sadasivan, 1958) will prove very instructive here. In India, wilt is confined to distinctly alkaline soils with a pH of 8–9. The addition of certain heavy metals such as boron, zinc, iron, and manganese is known to control wilt and there is evidence to show that the microbial numbers in the rhizosphere and in soil away from rhizosphere increase considerably in the presence of these amendments. It has further been shown that the addition of aluminum, lithium, boron, zinc, manganese, and iron to these soils also limits saprophytic survival of fusaria on plant debris. These facts indicate that reduction in pathogenicity which accompanies addition of these heavy metals to the soils is due at least in part to increased microbial antagonism to the pathogen in the soil and in the rhizosphere, although the possibility of increased host vigor and resistance cannot be ruled out. A point of further interest here is the controlling influence of pH and of combinations of these elements on their effectiveness in root disease control. With iron and manganese in 2 : 1 combination (40 p.p.m. iron : 20 p.p.m. manganese), for instance, maximum increase in microbial numbers in soils and greatest protection to cotton plants from wilt were both seen at pH 6. It is also known that microbial activity in these soils as well as saprophytic survival of *Fusarium vasinfectum* therein are interrelated and these, in turn, are modified by soil temperature, soil moisture, soil aeration, and also the nutrient status of the soil (Subramanian, 1950). That addition of organic nitrogen in the form of organic manure may also give protection to plants from wilt is evident from the work of McRae and Shaw on wilt of pigeon pea and of several workers on cotton wilt; even here the protection seen appears to be the result of increased microbial antagonism to the pathogens (Sadasivan and Subramanian, 1954).

In the case of soil-borne tobacco necrosis virus, the observation that host plants growing in bacteriologically sterile conditions and in water culture are not infected, but contract the disease in soil that has been recently sterilized, may be interpreted to mean that some members of the soil microflora serve as hosts for the virus; however, Bawden (1950) records that no experimental evidence for this hypothesis could be obtained.

Finally, it may be mentioned that synergistic effects between pathogens are also common and in many cases root diseases may be caused by mixed infections by more than one pathogen.

4. *The Rhizosphere: Pathogen in the Rhizosphere and Its Relation to Other Rhizosphere Microflora, the Microflora of the Soil, Root Exudates, etc.*

It is probably well to begin by defining rhizosphere. A soil ecologic region inside which the soil is subject to specific influence of plant roots is the rhizosphere; the credit for this definition goes to Hiltner (1904). It is a matter of surprise that the import of this impact of the growing root on the soil medium through which it grows failed to draw much attention until the thirties when Starkey (1929) revitalized the subject and did real creative thinking in this fascinating field of research. Since then, much work has been done and many excellent reviews on the subject have appeared (Katznelson *et al.*, 1948; Harley, 1948; Clark, 1949; Garrett, 1956). Consideration of the rhizosphere as a region of intense microbial activity would logically concede that the root surfaces are normally a more potent source of energy than the soil adjacent to plant roots and to designate this the term “rhizoplane” has been suggested which includes in its definition external surfaces of plant roots together with closely adhering particles of soil or debris. Other terms such as “outer rhizosphere” and “closer rhizosphere” have been used to designate sites of microbial concentration. Despite these newer terminologies, more recent investigations into many aspects of this complex problem of root exudates and microbial activity seem to favor and justify the use of just two terms, the rhizosphere to denote soil region adjacent to plant roots and the rhizoplane to indicate plant root surfaces.

There is, however, need for familiarity with one more technical term, the "rhizosphere effect," since on a proper understanding of it depends the quantitative assay of rhizosphere problems. The rhizosphere effect is the ratio of the number of organisms in the rhizosphere (which in our view includes the rhizoplane) and the number in the soil outside the rhizosphere, calculated on a soil dry weight basis. This is generally expressed as a positive effect if the ratio exceeds one, and negative if fractional.

Rhizosphere or rhizoplane microfloras and their relationship to production of antibiotics *in situ* have naturally broadened the field of inquiry and many new techniques have been evolved for detecting these products of microbial synthesis but it would appear futile to cover that ground, since our immediate task is to understand the rhizosphere in relation to root disease pathogens in as broad a sense of the term as possible. The building up of an active rhizosphere or rhizoplane in germinating seedlings does not take long and, indeed, Timonin (1940) showed that seedlings only 3 days old had 11 to 28 times as great a rhizosphere population as elsewhere in the same soil. It is now known that microorganisms seldom occur on the root tip and their appearance is governed by normal plant development rather than root growth. In other words, it is intimately connected with active and normal plant metabolism and, therefore, primarily on root exudations. Evidence indicates that this process is not a one way affair, in fact, the activities of microorganisms in the soil of the rhizosphere include decomposing of sloughed-off root caps, root hairs, cortical and epidermal cells, and making available organic and inorganic nutrients for absorption. The soil inhabitant class of organisms and the soil invaders have, therefore, an important part to play in these processes and together these floras constitute what one might designate as microecology in the broadest sense of the term reflecting as it does on the products of metabolic functions of the rhizoplane and the rhizosphere. Preliminary observations (Winter and Willeke, 1951a, b) on the properties of powerful antibiotics derived from the plants *Aucuba japonica* and *Myrtus communis* indicate their potential influence on the ecology and sociology of the microbial population of the rhizosphere.

Working with tropical soils, Agnihothrudu (1954) showed that the rhizosphere of the wilt-susceptible variety of pigeon pea, *Cajanus cajan* (Spreg.) Millsp. was more conducive to the survival of its causal agent, *Fusarium udum*, in contrast to the resistant varieties. Further, the actinomycetes antagonistic to *F. udum* were present in large numbers in the rhizosphere of the resistant varieties. The significance of this finding is becoming more obvious with recent investigations comparing the rhizosphere floras of resistant and susceptible varieties of crop plants.

It was shown earlier by Agnihothrudu (1953) that *Aspergillus* spp. predominated in the rhizospheres of potted plants, particularly *Sorghum dochna* var. *irungu* and cotton, from 15 days to 3 months after germination. In general, both in the unplanted soil and in the rhizosphere the percentage of *Aspergillus* spp. decreased toward the final estimation except that there was an increase in those plants that flowered, viz., *Phaseolus vulgaris*, *Cyamopsis tetragonoloba*, *Sesamum indicum*, and *Crotalaria juncea*. *Penicillium* spp. also increased gradually in numbers as the plants grew older. The changes in total numbers of fungi corresponded roughly to the changes in numbers and percentage of *Aspergillus* and *Penicillium*. *Fusarium* spp. were encountered regularly in the rhizosphere dilutions but very rarely in the control soil, the highest increase occurring with pigeon pea and the lowest with *S. dochna* var. *irungu*, possibly indicating an inhibitory root exudate in the case of sorghum. *Macrophomina phaseoli* and *Neocosmospora vasinfecta* were observed microscopically in the rhizoplane of all seedlings except sorghum but the organisms did not grow in dilution plates. In addition, eleven other genera of fungi were obtained from the rhizosphere, and in plants that flowered, there was a definite increase in the numbers and percentage present at the final estimation.

These results have found support in a more recent investigation into the rhizosphere floras of pea infected by *Fusarium oxysporum* f. *pisi* (Buxton, 1957a, b). Four pea cultivars which are differential hosts for the physiologic races of the pea wilt fungus *F. oxysporum* f. *pisi* exerted differing effects on the soil microflora. The cultivar susceptible to race 1 supported more fungi, bacteria, and actinomycetes near its root surface than do the cultivars that resist race 1. Spores of a race that can cause wilt to a particular cultivar germinate well in soil extract from that cultivar, whereas germination decreases in extracts from rhizospheres or in root exudates of a resistant cultivar. Similarly, the susceptible cultivar wilted severely where the susceptible cultivar was previously grown and inoculated with race 1, whereas wilting was less and developed more slowly when the cultivars resistant to race 1 had been previously cropped. Possibly, substances exuded by roots of cultivars resistant to race 1, prevent race 1 from germinating.

Sulochana (1958) has recently undertaken a quantitative study of the microfloras of the rhizosphere of cotton plants and has used reliable bioassays for analyzing exudates. The results are of far-reaching significance. The choice of soil was confined to the heavily wilt-sick soils from southern India where as many as fourteen species of pathogenic fusaria have been isolated before (Subramanian, 1951), the most predominant species being *Fusarium vasinfectum*. The rhizosphere floras of two genetic strains of two species of cotton (the diploid susceptible *Gossypium arboreum* race *indicum* L. and the tetraploid resistant *G. hirsutum* L.) were examined. In both species the amino acid requiring bacteria were qualitatively larger in numbers than the vitamin requiring bacteria. Quantitatively, higher numbers of both groups of bacteria existed in the rhizosphere of diploid strains than that of the tetraploid strains. The bacterial numbers of both groups diminished as a result of the rhizosphere effects of wilting susceptible diploid plants in soil when the pathogen was present. This indicates that much of the energy substances exuded had been utilized by the pathogen *F. vasinfectum.* Quantitative assays for amino acid were made using the test organisms *Lactobacillus arabinosus* 17/5 and *Leuconostoc mesenteroides* P 60. Concentrations of amino acids were higher in the exudates of diploid strains than the tetraploid strains. Assays of the vitamin B group using X-ray mutants of *Neurospora crassa* and *N. sitophila* indicated that vitamin B concentrations were considerably higher in the vicinity of the diploid than of the tetraploid plants. In general, exudation of amino acids and vitamins could be directly correlated with the rhizosphere activity. On growing susceptible diploid variety of cotton in garden soil at constant temperatures of 32.5°, 35.0° and 37.5° C., no appreciable effect in the rhizosphere of healthy and infected plants was observed at the two higher temperatures. This is not surprising. At these high temperatures an abnormal host metabolism could result in a minimum of exudation of energy substances utilizable by the rhizosphere microorganisms. This could be the deciding factor in the observed marked reduction on both the amino acid and the vitamin requiring groups of bacteria. The energy material exuding from the root systems of genetically differing Old and New World cottons may well form the springboard for the development of saprophytic antagonistic bacterial floras and of mycofloras as well. These form part of the rhizosphere flora and are known to act as root-infecting pathogens. We have not examined sufficiently the implications of gene controlled mechanisms in light of how they affect uptake and exudation of metabolites. Quite obviously, the plasma membranes of root systems are worth studying more critically since in them rests the key to exudates and their repercussions on saprophytic and parasitic microfloras of the rhizosphere and, more particularly, the rhizoplane. Apart from exudations of amino acids and sugars, substances of large and small molecular weights such as nucleotides and flavanones have been recorded from pea exudates (Lundegårdh and Stenlid, 1944; Fries and Forsman, 1951).

Studies on the nature of root exudates of many crop plants under varying soil conditions have been undertaken by numerous workers.

Katznelson *et al.* (1954) showed that desiccation and subsequent rewetting of the sand in which tomatoes, soybean, barley, or oats grew, resulted in the excretion of glutamic acid, aspartic acid, leucine, alanine, cysteine, glycine, lysine, phenylalanine, proline, and a reducing compound of R_f value identical with glucose. Rovira (1956a) indicated that, under aseptic growing conditions in quartz sand, peas excreted twenty-two different amino acids while oats excreted fourteen of them. He further indicated (Rovira, 1956b) that addition of exudates from the roots of peas and oats *in vitro* increases the growth of microorganisms isolated both from control soil and the rhizosphere of 3-week old pea plants. This stimulation was greater for organisms from the rhizosphere than for those from outside this zone. Some organisms responded in a comparable way to addition of yeast extract, whereas others did so with exudate plus yeast extract. The root exudate was not totally replaceable by glucose, soil extract, vitamin free casamino acids, or a synthetic mixture of growth factors. Stimulating action of yeast extract on microorganisms is generally considered to be due to unidentified factors.

Bhuvaneswari and Subba-Rao (1957) examined the root exudates of *Sorghum vulgare* var. *dochna* and *Brassica juncea* and spotted several organic acids and sugars in them. Although tartaric and oxalic acids, D-xylose and D-fructose were common to both plants, *B. juncea* had malic and citric acids, D-glucose and maltose in addition. The possibility of malic and citric acids as factors influencing the observed depression of the microflora of *B. juncea* has been suggested by these authors. Root exudates of paddy infected by the foot rot organism, *Fusarium moniliforme*, have been the subject of study recently (Andal *et al.*, 1956). Chromatographic analysis of exudates from susceptible and resistant strains of paddy under inoculation revealed that aspartic acid, glutamic acid, tryptophan, and lysine were common to both plants and occurred in almost the same concentrations. In addition, the resistant strain had cystine, asparagine, tyrosine, and methionine. Especially the sulfur containing amino acid cystine has been shown in this laboratory to be present in fairly large quantities in the roots of the tetraploid variety of cotton, *Gossypium hirsutum* L., which is resistant to wilt caused by *Fusarium vasinfectum* (Lakshminarayanan, 1955). Studies of the rhizosphere microfloras of these two strains of paddy showing differing amino acid exudation would be of considerable interest.

An interesting example of possible root exudation and its effect on pathogenic fungi comes from the work of Ellis (1951) who demonstrated that the clubroot fungus (*Plasmodiophora brassicae*) was greatly reduced or was eliminated when susceptible cabbages were grown after peppermint (*Mentha piperita*) had been cropped from 1 to 3 years.

The author concludes that the stolon of *M. piperita* was perhaps indirectly responsible for the production of substances antagonistic to *P. brassicae*. On the other hand, there may be direct correlation between exudation from *M. piperita* and *P. brassicae* antagonism. This problem can, therefore, be pursued further. Root exudates of *M. piperita* could be examined for their ability to cause widespread soil disinfection of the otherwise persistent pathogen.

There are also other functional advantages that rhizosphere microfloras can confer in the normal uptake and utilization of inorganic salts by roots from soils. Gerretsen (1948) reported that roots of oats, mustard, sunflower, and rape with a rhizosphere population were capable of absorbing and utilizing insoluble mineral phosphates which were only slightly available to sterile roots. There is also evidence from other sources that, by absorbing the water-soluble phosphorus compounds, the bacteria prevent to a certain extent the chemical decomposition of these compounds in soil. Varieties of oats susceptible to manganese deficiency supported greater numbers of microorganisms that showed capacity for oxidizing manganese into unavailable forms than the resistant varieties (Timonin, 1947). Conversely, microorganisms have been found to reduce availability of microelements when energy materials are added to soils. Eliminating the natural microflora by sterilization of soils cures certain microelement deficiency symptoms, the effect being a direct consequence of removing the microbes that act as competitors for the meager supply of micronutrients essential for normal plant growth.

There may be many other functional mechanisms of ionic uptake by higher plants in both acidic and alkaline soils, not only of macro- but of microelements also, where the role of microorganisms has to be determined by careful experimentation before a verdict is given. In this, much ingenuity in evolving techniques has to be displayed, particularly in the collection of root exudates under aseptic conditions. Bioassays must be more widely developed for these investigations. Without them, the dynamics of the rhizosphere and rhizoplane could hardly be expected to progress beyond a stage of stalemate.

D. *Root Infections and Symbiosis: Mycorrhiza, Root Nodule Bacteria*

We cannot accept that all infections of roots that are beneficial to their hosts are mycorrhizal or that the term "mycorrhiza" specifically pertains to proved examples of symbiosis. Both aseptate and septate mycelial forms of great diversity enter into this symbiotic relationship with rhizoids and roots of bryophytes, pteridophytes, and angiosperms. Collectively, these constitute the endotrophic mycorrhizas as opposed to the ectotrophic that have mycelium external to the roots. The ectotrophic form has a well developed sheath of fungal pseudoparenchyma enclosing the root, the fungal hyphae penetrating between cortical cells, but few entering them. By contrast, in the endotrophic form the fungus is in the tissues, particularly in the cortex where the host protoplasm digests it. The aseptate fungi produce both arbuscules and vesicles that aid the digestion of the host tissues. The type of digestion varies from thamniscophagy (arbuscule and sporangiole digestion) to tolypophagy (formation of digestion clumps). The terms mycorrhiza and pseudomycorrhiza are at present not precisely definable. Infected roots showing ectotrophic mycorrhizas, as in the case of forest trees, exhibit constant morphological form, i.e., presence of external mantle and internal Hartig net (the central core of host tissue which is penetrated by the fungal hyphae in its outer cortex growing among the cells to form a network is the Hartig net, named after the German botanist), hypertrophy of the cortex, and characteristic branching. The pseudomycorrhiza covers a wide variety of endotrophic and ectotrophic associations which depart in morphological form or physiological function from the typical cases. In other words, the pseudomycorrhizal as well as the mycorrhizal ectotrophic forms show a microorganismal population in the rhizoplane and the rhizosphere in which one or a few fungi dominate in functional activity. For more detailed treatment of this and related literature, the reader could most usefully study the splendid reviews on the subject by Harley (1948, 1950, 1952).

The occurrence of digestion *in vivo* in the typical endotrophic forms no doubt demonstrates an exchange of material between the host and the fungus but its absence does not prove to the contrary, indeed, there are other modes of exchange that need not be preceded by digestion. The degree of dependence of the fungus on the soil for its vital metabolic needs in the shape of organic and inorganic nutrilites has not been precisely defined. However, it is generally understood that the penetration and infection of young roots by the fungus is from the soil or from plant residues or by root contact.

Many members of the Agaricaceae, Boletaceae, *Scleroderma*, etc., form mycorrhizas. Recent investigations by Robertson (1954) have shown that some of these produce air-borne basidiospores, e.g., *Boletus granulatus*.

Apart from the theoretical aspects of mycorrhizal infection and their importance in plant growth, the problem is one of great practical application, when fully understood. In the presence of appropriate fungi, under artificial inoculation conditions, there is undoubtedly great increase in growth of seedlings. The probable mechanisms involved in this increase are: that the fungi may alter the insoluble inorganic carbon compounds into soluble form, or change the pH in the culture medium, or produce vitamin-like substances (auxins?). This faculty is not confined to mycorrhizal fungi, however.

There is yet no proof that infection of the root tissue and the growth of the endophyte into the tissue are essential for host stimulation and many believe that infection is purely incidental. (The dependence of seeds on biological stimulation, whatever its mechanism, does not appear to offer an explanation for the formation of mycorrhiza in later stages.) Nevertheless, it is difficult to discountenance the need for an external food base wherefrom the fungus would derive sufficient energy to enable it to develop into an active endophyte. The ability of endophytes to grow *in vitro* does not in any way indicate that there exists a competition for organic matter in soil. Burges (1936) states that infection is unimportant and the external activity of fungi is all important in the nutrition of mycorrhizal hosts. It would then appear that the emphasis almost completely shifts to the life of the endophyte in the soil, particularly in the rhizosphere and the rhizoplane. The problem of the endotrophic mycorrhiza, therefore, appears to be one of great complexity, where the food base in the soil from which the fungus meets its energy requirements and the conditions under which it parts with it to host tissue *in vivo* are logical steps in experimentally elucidating the sequence in the process. The main point to appreciate in the case of the ectotrophic mycorrhizas, on the other hand, is that a high proportion of absorbed materials must necessarily enter the infected roots routed through the fungal sheath as, indeed, the fungal sheath is a living entity with powers of selective absorption. The completeness of the living fungal sheath and its intimate connection with the root cortex in mycorrhizal associations are of paramount importance in planning further experimentation to understand this functional physiology.

Apart from these general considerations, a number of fundamental investigations on the physiology of the mycorrhiza needs discussion in the present context. Harley and McCready (1952a, b) examined excised mycorrhizal root tips of beech, separating the fungal sheath from the core after absorbing radioactive phosphorus (P^{32}) from aerated media at pH 5.5. Approximately 90% of the phosphorus accumulated in the fungal sheath. No significant change in the ratio occurred over a 24-hour period using a concentration of 1 mg. per liter. Cores freed from their sheaths before exposure to phosphate absorbed phosphorus four times more quickly than the cores of intact tips. Harley and Brierley (1954) further showed that the route by which P^{32} passes through the fungal tissue into the host tissues of beech mycorrhiza was from the sheath into the core. The rate of movement of P^{32} from fungus to host was rapid at first but became slower after 15 hours and the rate was temperature sensitive, becoming very slow at 1° C. The process of transport was sensitive to oxygen concentration, slackening very much when oxygen concentration in solution was below 3%. It was obvious that active transport of phosphorus from fungus to host occurs in mycorrhizal beech roots and the mechanism of transport is dependent on aerobic metabolic processes in the fungal tissue as well as on the absorptive processes of the core. Phosphate lost from roots kept in low oxygen concentrations is entirely released from the sheath and this results from temperature sensitive anaerobic processes occurring in that tissue.

Studying phosphate uptake further, using P^{32} (Harley and Brierley, 1955), it was shown that excised mycorrhizal roots of the beech when washed in buffer solution containing phosphate recorded reduced rate of transport from fungus to host. When roots had been returned to phosphate free buffer, after a period in buffer containing phosphate, the temporarily reduced active transport was resumed at a rapid rate. It is suggested that the phosphate absorbed from the external solution competes successfully for a substance produced in respiratory metabolism so that phosphate accumulated in the sheath remained immobilized. In the absence of external phosphate supply, the phosphate already accumulated in the sheath was utilized. Movement of phosphorus into the core was mainly from the external solution when this contained phosphate, but was from the sheath phosphate when the external supply failed. Earlier, Harley and McCready (1952a, b) had shown that the fungal sheath constituted about 39% of the dry weight of mycorrhizal roots of beech. The sheath of intact mycorrhizas restricted the uptake of phosphorus by the cores—and this reduction was less at higher than at lower concentrations—acting as a partial barrier to diffusion and phosphorus absorption seemed to be linked with metabolic activity over the whole range of phosphorus concentrations studied. There are two possible routes by which phosphorus may reach the core by diffusion through the intercellular spaces and cell walls of the sheath and by way of the living sheath cells.

That the physiological state of metabolism of the plant has much to do with mycorrhizal function finds support from the work of Harley and Waid (1955). The influence of different levels of daylight radiation on the growth and nature of mycorrhizal infection of beech seedlings was direct, the mycorrhizal infections on the root systems appearing after the first true leaves emerged. Plants receiving more light showed more vigorous development than shaded plants. Shading eventually led to a loss of resistance by the beech seedlings to parasitic infection while increases in light intensity resulted in mycorrhizal formation.

There is yet another aspect of nutritional requirement by the mycorrhiza-forming fungi. Melin (1954) tested a number of Hymenomycetes and Gasteromycetes and all of them were partially or totally deficient in thiamine. Most species were heterotrophic for both the thiazole and pyrimidine fractions while five species of *Tricholoma* were more deficient in pyrimidine than in thiazole. Some species of *Cortinarius* had a reduced capacity for synthesizing thiamine from its two components, thiazole and pyrimidine. Most Basidiomycetes greatly benefited by the presence of small amounts of amino acids in ammonium nitrogen containing medium. The main sources of these nutritional substances in nature seem to be the soil and not the roots. *Boletus* spp. grow extremely well *in vitro* on excised *Pinus sylvestris* roots in a nutrient solution supplemented by amino acids plus the B vitamins, whereas without roots growth was barely visible. Pine roots seem to produce one or more growth-promoting metabolites essential for the growth of tree mycorrhizal fungi which are deficient in these substances. Excised tomato roots and germinating pine seeds affect the growth of these fungi in the same way as pine roots, indicating that the metabolite is not specific to pine roots.

Much has been said to indicate the somewhat parallel nature of the problems of the saprophytic and parasitic forms of the rhizosphere and the mycorrhizal habit as far as the interdependence of host metabolism is concerned for both types of infections. Quite obviously, much of the energy substances exuding from roots are utilizable by the micropopulation and it is probably premature to draw a line and compartmentally assign these microbial functional processes. There seems little doubt, however, that this twin field of research would give endless opportunities for more critical work on absorption and movement of metabolites from the soil into the plant tissues and back as exudates and their utilization by facile endotrophic and ectotrophic living microbial populations.

The root nodules of Leguminosae and the bacteria that partake in this symbiotic relationship present another interesting facet of soil microbiology. As early as 1587, Dalechamps named a species *Ornithopodium tuberosum* which could be distinguished by its nodule-bearing habit. The practical significance of these nodules was first noted by Hellriegel in 1886 when he mentioned that nitrogen fixation in the leguminous plant occurred only when root nodules were present. Since then the leguminous nodules have been studied intensively by many workers (Allen and Allen, 1954; Thornton, 1952, 1954) and it will be pertinent here to summarize briefly our present knowledge of this subject.

The general assumption that nodulating ability is characteristic of all Leguminosae is probably not correct. Indeed, only less than one-half of the genera in this order have been examined and of these many species falling under Caesalpinioideae do not possess the ability to form nodules. The greatest number of species having this ability belong to the subfamily Papilionatae. Compatibility of the bacterial and plant proteins, and a direct relationship with the calcium fraction of the leguminous plants (this fraction being greater in leguminous than in nonleguminous plants) have been suggested to explain why rhizobia produce nodules only on leguminous plants. It has also been pointed out that the leguminous plant produces an enzyme which enables it to select, entrap, and use the particular form of organic nitrogen contained in the invading rhizobia. Several workers have also recently demonstrated resistance to nodulation by pure lines of genetically selected red clover (Nutman, 1946, 1949) and soy bean (Lynch and Sears, 1952; Williams and Lynch, 1954) which indicates that a recessive hereditary factor in the plant may also be involved.

The rhizobia are facultative parasites but their role in symbiotic nitrogen fixation has led to their being regarded as symbionts. Culturally, all strains are aerobic, heterotrophic, gram-negative rods. Although soil is the normal habitat of these bacteria, it is extremely difficult to distinguish them from other soil bacteria such as *Radiobacter* by ordinary methods. They are normal members of the soil microflora and can exist for many years in field soil without their host plant. However, its presence brings about a definite increase in the number of rhizobia in the neighborhood and this is possibly due to stimulatory root secretions (Thornton, 1929). Strains differ in the rates at which they multiply around roots under the influence of these secretions (Nicol and Thornton, 1941). It is difficult to estimate the number of nodule bacteria in a natural soil, since the only definite test for *Rhizobium* is its ability to produce nodules on its host plant. However, approximate estimates of numbers obtained by supplying the plants with serial dilutions of the soil sample and finding out the extent of infection, were extremely variable, but these indicated that the numbers, for instance, of clover *Rhizobium* may be of the order of tens of thousands per gram in a soil of pH suitable for clover. Diversity of strains in a given soil is common. Proof is wanting as to the ability of rhizobia to fix atmospheric nitrogen in the absence of the host plant, in the soil, and in pure and mixed cultures *in vitro*.

In the understanding of the physiological relationships between the rhizobia and the host plants, two terms require definition: (1) infectiveness and (2) effectiveness. The former connotes ability of a strain to cause the formation of nodules on certain leguminous species and not on others; the latter, the ability of a strain to help the growth of the plant by nitrogen fixation. In each of these, various gradations in relationship also occur. *Effective nodules* fix quantities of nitrogen normally adequate for the plant's needs, whereas *ineffective nodules* fix little or no nitrogen. These two types of nodules are known to show marked differences in the course of their development. In both types, early stages of growth are similar, the nodule first consisting of a tiny mass of meristem cells usually derived from the root cortex. Most of the central cells become infected and cease to divide. These cells are surrounded by a layer of uninfected cells which remain meristematic and form a distal cap. Owing to the activity of these meristematic cells, the nodule grows and the inner layers of newly formed cells are successfully invaded by bacteria. Vascular strands are later formed connecting the nodule with the central cylinder of the root; a secondary endodermis also appears. From about this stage, the two types of nodules differ. In the case of effective nodules, further growth takes place leading to the formation of a conspicuous mass of central bacterial tissue; in the case of ineffective nodules both the meristem and the central bacterial tissue are transient. In the latter case, the bacteria become parasitic on the host tissue and cause its disintegration and, indeed, this necrotic process extends distally through the central tissues until the middle of the nodule is destroyed and it ceases to function. In the case of the effective nodule, the central bacterial tissue is the seat of the symbiotic nitrogen fixation process. Further, it contains four well-defined pigments: leghemoglobin (red), legcholeglobin (green), legmethemoglobin (brown) and coproporphyrin (brown). Leghemoglobin is markedly similar to hemoglobin of blood and a molecular weight of about 34,000 (i.e., about half that of blood hemoglobin) has been reported for a preparation which approximated 85% purity; it was first identified by Kubo in 1939 and later confirmed by Keilin and Wang in 1945. Leghemoglobin does not occur in detectable amounts in ineffective nodules.

The function of hemoglobin and its derivatives in nodules remains an unsolved and intriguing problem, although there is circumstantial evidence connecting hemoglobin with nitrogen fixation. Neither the plants nor the rhizobia produce the hemoglobin independently, and it occurs only during the stage of effective symbiosis. It would appear that it is a product of the *rhizobium*-leguminous plant complex, one agent presumably contributing the hemin fraction and the other the protein fraction of the molecule. Its presence in effective nodules is closely linked with the photosynthetic activity of the host plant. In mature effective nodules it first appears during the differentiation of the bacterial tissue and can be detected histochemically only in rhizobia-packed cells; proof is wanting for its occurrence within the rhizobial cell per se, in the uninfected cells of the bacterial tissue, in the nodule cortex or within the meristematic area. Further, free-living rhizobia are not able to fix nitrogen following addition of hemoglobin and purified leghemoglobin. A further noteworthy fact is that no hemoglobin is produced in non-symbiotic nitrogen-fixing systems. Space does not permit a detailed discussion of the biochemical aspects of symbiotic nitrogen fixation for which the reader should refer to the excellent reviews of Thornton (1952, 1954) and of Allen and Allen (1954).

Nutman (1946) has proposed the terms "responsive" and "unresponsive" for plant reactions to nodulation determined by genetic factors. However, it is far from clear whether the differences in plant response are due to the inherent qualities of the bacterial strains, to conditions within nodules, or to the influence of the host itself.

Infection of the leguminous root usually takes place through root hairs, although entry may be effected through epidermal and cortical cells and also through ruptured tissue at the points of lateral rootlet emergence. In the case of *Neptunia oleracea,* an aquatic leguminous plant lacking root hairs, epidermal cells appear to be the sole points of entry. Normally, a colony forms near the tip of a root hair; the latter excretes a substance (β-indolylacetic acid ?) which causes the root hair to curve and at the bent tip the bacteria make way through the cell walls into the root hair. They then pass into the cells of the root, multiply rapidly and form the nodule. The number of nodules produced is characteristic for each bacterial strain, different strains exhibiting differences in infectivity. With a given strain and host plant, there is, nevertheless, correlation between the dose of bacteria supplied to the root region, the number of root hairs infected, and the number of nodules produced. Normally, host root systems may be simultaneously invaded by several strains of *Rhizobium* and different strains can be isolated from nodules on the same plant in the field. In the presence of more than one strain, the proportion of nodules produced by each strain will depend on (a) the relative numbers of each strain in the root region and (b) the relative infectivity of the strains. Nodulation produced by one strain may saturate the nodule-forming capacity of the host and when this happens a second strain may be excluded, although it cannot be concluded that invasion by one strain confers immunity to the host from subsequent invasion by a different strain or strains.

Much more remains to be known about the mechanism of rhizobial entry into the root. Earlier suggestions about the secretion of the enzyme cytase by the rhizobia and consequent dissolution of the root hair wall lack experimental proof. It would appear that the curling of the hair is due to some secretion produced by the rhizobia before they enter. Thornton (1929) associated infection with production of a stimulatory substance by the roots at the time the first leaf unfolds. During the period between germination and opening of the first true leaf of alfalfa, no infection occurred, whereas one day later 2% of the hairs were infected. Addition of the sterile extracts of solutions surrounding the roots of seedlings bearing first leaves to younger seedlings enhanced root hair infections. Production of the stimulatory substance is probably controlled or influenced by the top of the plant, although removal of the first leaves does not apparently delay nodulation. Moreover, nodules may be formed on excised roots.

Nutman (1952) reported that with either lucerne or clover the actual numbers of nodules are greater on plants growing singly than on plants growing in pairs or in large groups within a culture of standard size. This inhibition of nodulation on plants growing together appears to be due to the diffusion of some substance from the roots, since the extent of the inhibition depends on the volume of the medium as well as the number of seedlings present. The number of infections per plant is directly proportional to the volume of the medium and inversely proportional to the density of planting. Turner's (1955) more recent work on nodulation in clover plants further supports Nutman's findings. It has been shown that the addition of charcoal to the rooting medium of clover plants inoculated with effective and ineffective strains of *Rhizobium* leads to a stimulation in nodule formation. In the presence of charcoal, the period between inoculation and the first appearance of nodules is reduced. One possible explanation of this phenomenon is that the stimulation is due to the adsorption by the charcoal of inhibitory compounds secreted by clover roots. These compounds have been eluted from charcoal and have been shown to influence nodule formation. Indeed, Virtanen and Laine (1939) have shown that aspartic acid, β-alanine, oxime N, and fumaric acid may be secreted from the nodule-bearing roots of clover plants which are actively fixing nitrogen. Glutamic acid has also been detected (Virtanen, 1948). The influence of these secretions on nodulation is not known. However, repeated cultivation of clover crops in the same soil may lead to "clover sickness" which may be due to the accumulation of toxic compounds in the soil secreted by the clover plants; such sickness can be overcome by addition of charcoal to soil.

Apart from root excretions influencing nodulation, certain other factors may also have similar effects on nodule formation. For instance, invasion is often governed by the carbohydrate-nitrogen balance in the root hair. Thus, seedlings of etiolated vetch and albino soybean and *Leucaena* are not able to produce nodules unless an adequate amount of carbohydrate is supplied. According to Nutman (1946), resistance to infection of red clover by *Rhizobium* is attributable to a hereditary factor which delays nodulation as late as the unfolding of the fifth or sixth leaf. Results of experiments involving grafting of genetically susceptible clover tops onto roots of resistant plants and vice versa suggested no translocation of substances influencing infection (Nutman, 1949).

The relation of the general soil microflora to nodulation has been well brought out by the work of Harris (1953) who investigated the plant-rhizobial relationships between *Trifolium subterraneum* and strains of *Rhizobium trifolii* in pot experiments. One strain (No. 44) was found to give an ineffective reaction (as measured by plant growth) in heat sterilized soils, whereas in similar nontreated soils an effective reaction was seen. In order to study the effect of associated fungi on nodulation, sterilized subterranean clover embryos were grown asceptically on a mineral salts soft agar medium free from nitrogen and carbohydrates. When the first trifoliate leaf had unfolded, the seedlings were inoculated simultaneously with *R. trifolii* and a suspension of one of the fungi frequently isolated from clover roots in nonsterile soils. The presence of a pathogen capable of killing the plant rapidly inhibited nodulation completely, as in the case of *Fusarium* 1013 and *Sclerotinia* 1015. Where the degree of pathogenicity was less marked but root invasion occurred in the extensive local root rots, as in the case of the dematiaceous fungus 1017 and *Hormodendrum N*, nodulation with strain 430 of *Rhizobium trifolii* was reduced, probably owing to impaired metabolism in the plant rather than direct antagonism of the fungus to rhizobia. Some strains of fungi and bacteria stimulated the weakly virulent strain 44, but had no effect on the more active strain of the bacterium.

These results point to the need for more comprehensive and intensive studies on the same lines and experimental work aimed at understanding the rhizosphere effect *vis-a-vis* root exudates in nodule-forming leguminous plants would, indeed, be a most fruitous path for future studies. There is no doubt that the leguminous root nodule still poses many interesting and intriguing problems for the experimental microbiologist.

IV. PATHOGEN-HOST RELATIONSHIP

The essential features of the pathogen-host relationships seen among root-infecting pathogens may now be considered. If one is guided by the damage caused to root systems or to the plant itself via root systems by organisms, it would appear erroneous to leave out of consideration the large number of organisms which are known to produce antibiotics, toxins, and other metabolites in soils which might predispose root systems to infection. However, it is necessary to maintain a distinction between such damage brought about by metabolic products of microbes and damage following infection by a pathogen. In what follows, therefore, the discussion will be centered on the pathogen in its relation to the host.

It is probably difficult to state which is the predominant factor in this dual relationship, the pathogen or the host. Nevertheless, it is safe to assume that the host, its rhizoplane, and its rhizosphere merely provide the substrate for the activity of the pathogen and, in the absence of the latter, there is neither infection nor pathogenesis. Pathogenesis, of course, implies that a pathogen must effect entry into the host and assimilate the available nutrients, must tolerate or overcome host resistance, and induce disease in the host by its action on host tissues.

What appears to be a simple type of relationship is that seen among some pathogens of the destructive type which kill the host tissues and derive nourishment from the tissues so killed. This behavior is characteristic of many facultative parasites and the whole course of events leading to pathogenesis would appear to rest on the ability of these pathogens to secrete enzymes capable of dissolving the host substrate and killing the cells. Enzyme studies on several root rot, soft rot, and damping-off pathogens have shown, for instance, that the three pectolytic enzymes, polygalacturonase, depolymerase, and pectin methyl esterase are invariably produced by several of these organisms. It is reasonable to expect in such cases correlation of pathogenicity with presence and amount of the enzymes produced. No doubt, such a correlation has been reported in some cases, but not always. In studies on various species of *Rhizopus*, which are wound pathogens causing rot in sweet potatoes, for example, some of the pathogenic species (*R. nigricans* and *R. artocarpi*) secrete less pectinase than the two nonpathogenic species (*R. chinensis* and *R. microsporus*). Further, one species which produces the largest amounts of the enzyme is not pathogenic to sweet potato. Similarly, it is difficult to explain why *Botrytis cinerea*, which rarely parasitizes potato, should be able to produce active enzymes in potato decoctions of various strengths, whereas *Pythium debaryanum*, which is pathogenic on ordinary mature potato tubers, shows negligible amount of enzyme secretion on ordinary potato decoctions. It is obvious that there are differences between the pectinase enzymes of *Botrytis* and of *Pythium* and, as suggested by Brown (1948), "the interpretation of these differences may be that the enzymes are different in themselves or that they are the same but that some of their properties are conditioned by other metabolites produced by the pathogenic fungi."

Apart from dissolution of cell walls, a feature characteristically seen in some of these primitive pathogens (e.g., *Botrytis cinerea* and *Sclerotinia sclerotiorum*), is their ability to increase considerably the permeability to water of the host cells just beyond the discolored necrotic zone. Some substance other than the pectinase may bring about this change.

The pathogen-host relationship in common scab of potato due to *Streptomyces scabies* presents some noteworthy features. Following penetration through lenticels, stomates, wounds, or directly through the cuticle when it is thin, in young tubers of susceptible varieties many layers of dead cells are formed on the exterior in which the pathogen develops as a saprophyte. The underlying living cells then undergo abnormally rapid division and there is consequently sloughing off of more dead cells on which the pathogen continues to grow. It would appear that there is little invasion of living tissue and, indeed, the scab lesion results from proliferation of the latter. The mechanism of cell proliferation remains obscure. In resistant tubers, few dead cells occur at the surface and, therefore, the pathogen is not able to establish itself (see Walker, 1957). Another interesting fact is that peel extracts from tubers examined chromatographically or by taking ultraviolet absorption curves showed a much higher concentration of chlorogenic acid in a resistant than in a susceptible variety. In some resistant varieties, the chlorogenic acid appeared to be more heavily concentrated in the periderm and particularly near the lenticels, which serve as natural avenues of entry for the pathogen, and also around mechanical or parasitic injuries (Johnson and Schaal, 1952).

Whitney's studies (1954) on *Rhizoctonia crocorum* indicate that its ability to parasitize carrots requires substantial saprophytic development prior to infection. Its behavior, therefore, is somewhat comparable to that of *Streptomyces scabies*. The fungus infects mainly dicot hosts with roots which develop periderm tissue at some stage in their ontogeny. The susceptible stage of the carrot corresponds with that at which the carrot sheds its cortex in favor of a periderm. In such hosts, therefore, the pathogen can develop initially as a saprophyte on the periderm as a result of which it acquires the ability to launch a parasitic attack on the underlying tissues. In the invaded carrot tissue, intracellular sclerotial bodies are formed which appear to give rise ultimately to minute infection bodies. The intracellular hyphae are not haustorial and do not establish a nutritional balance within the invaded cell; on the other hand, the invaded cells are killed. In the organized host, conditions are not favorable for the more rapid fungus growth that usually occurs during its saprophytic development on agar and, therefore, under such conditions when nutrients are exhausted, the pathogen goes into a resting stage forming internal sclerotia.

The vascular wilt diseases involve quite a different type of relationship between pathogen and host. In the typical cases as, for instance, in fusariose wilts of cotton and tomato, following infection, the pathogen reaches the xylem and pervades this tissue and appears to cause little disintegration of the root systems and other living tissues. The complicity of enzymes, vivotoxins, and vessel plugging in pathogenesis of these plant wilts has been discussed earlier and by a number of authors (Dimond and Waggoner, 1953b; Subramanian, 1955; Walker, 1957; Sadasivan, 1958) and it would be superfluous to cover the ground again. Attention may, however, be called to the fact that among pathogenic forms of *Fusarium oxysporum*, most of which produce vascular wilts, some appear to produce cortical decay also. For instance, in watermelon wilt, at low temperatures, *F. oxysporum* f. *niveum* causes preemergence damping-off. This is a good example of the effect of the environment in modifying the pathogen-host relationship.

In the case of dry rot of potatoes caused by *F. avenaceum* and *F. coeruleum*, the latter grows through the intercellular spaces, the adjacent cells remaining alive, often for considerable periods—a situation somewhat suggestive of balanced parasitism. *F. avenaceum*, on the other hand, kills and penetrates the cells with which it comes in contact (McKee, 1954). This may be compared with what happens when blight-resistant potato tubers are inoculated with virulent and avirulent strains of *Phytophthora infestans*. Avirulent strains cause a rapid necrosis of the tissues with which they come in contact and are, thus, prevented from further spread or fructification; necrosis, however, is less rapid in tissues infected by virulent strains which are, therefore, able to spread through the tuber and fructify. These findings illustrate clearly the tendency exhibited by some pathogens to form a somewhat balanced association with the host. There is, of course, no question of mutual benefit here and it is a one-way traffic detrimental to the host. A greater approximation to balanced parasitism than what is seen in the case of *Fusarium coeruleum* is what characterizes the vascular wilt fusaria already referred to.

Some of the soil-borne smuts certainly exhibit a much better adaptation to live in a balanced association with their hosts. *Tilletia tritici* infecting wheat plants and *Ustilago violacea* infecting plants belonging to the Caryophyllaceae may be cited here. In both, seedling infection is common. In the case of *Tilletia tritici*, germination of brandspores in soil is followed by penetration into the seedling. Following penetration, the hyphae grow intercellularly to the growing point and develop immediately behind it throughout the vegetative period. Later, the young leaves are invaded from the growing point, but only some mild symptoms appear at this stage. In the final stages, the flower primordia are invaded; the embryo is destroyed, the endosperm tissue is attacked, and spores of the pathogen are then produced in enormous numbers within the seed. The sequence of events in the case of *Ustilago violacea* is similar, except that this pathogen ultimately affects the anthers and produces its spores within them. What is unique about both these diseases is the prolonged balanced association between pathogen and host from the seedling to the flowering stage.

The classical examples of true balanced parasitism are, of course, the rusts, and it is noteworthy that root infections by this group of pathogens appear to be very rare. Probably the only example of root infection by a nonsystemic rust is that recently reported in the case of *Puccinia carthami* which causes a foot and root disease of safflower (Schuster and Christiansen, 1952).

We may now consider cases of root infection which result in marked hypertrophy or hyperplasia of host tissues. Clubroot of crucifers, potato wart, crown gall, hairy root, etc. are common examples. Crown gall, which is caused by *Bacterium tumefaciens,* has been studied intensively by a number of workers. This and other hyperplastic diseases are discussed in Chapter 6 of Volume I.

The mechanism of action of other root-infecting pathogens causing hyperplastic diseases, such as *Plasmodiophora,* has not been investigated in detail. In the case of potato wart, differences have been reported between warted and sound tubers; for example, the pH of the warted tissue is claimed to be higher than that of healthy tissue. Moreover, quantitative data on the ash content of healthy and warted tissue showed greater amounts of mineral constituents in diseased tissue, particularly of iron, manganese, copper, and nitrogen—an observation which probably indicates that the stimulus to hypertrophy results from the diversion of these substances to the seat of infection.

Mention may also be made here of some interesting observations on associations between plant roots and viruses (see Bawden, 1950). In some cases, such as the phony disease of peach, viruses appear to be localized within root systems without producing, however, any visible symptoms. On the other hand, in wound tumor disease, the roots have many spherical woody tumors which vary in size in different hosts; the largest tumors are formed in sweet clover plants *(Melilotus alba* and *M. officinalis)* and in *Rumex acetosa.* Wounding appears to be essential for the initiation of these tumors. Moreover, these tumors have been shown to be capable of indefinite growth as tumor tissue. In the little known clubroot disease of tobacco, the distortion of smaller and larger roots, closely similar to clubroot of cabbage, is the main symptom. Death of lateral rootlets is common in some tree diseases of virus origin, e.g., elm phloem necrosis. Death of lateral roots is also a common secondary symptom in tristeza disease of citrus, the roots being starved due to interruption of normal food transport. The disease occurs only when sweet orange scions are grafted on to sour orange stocks; the trees, however, hardly show symptoms characteristic of virus infections and they apparently die from a root rot. This rotting of roots appears to be a secondary effect resulting from the death of the phloem cells at the stock-scion junction and the consequent interruption in translocation of food from scion to stock. The sweet orange apparently is a carrier of the virus to which the sour orange is hypersensitive. When infected, the scion manifests no symptoms; movement of the virus into the stock, however, causes local reactions of a necrotic type which interfere with translocation of food, so that ultimately the whole tree dies (see Bawden, 1950).

From what has been stated it will be evident that the subject of pathogen-host relationship, as far as it relates to root-infecting pathogens, is a complex one, involving as it does interactions between the soil, the pathogen, and the host. No doubt, the facultative parasite has received more attention in studies of relationship between pathogen and host, but our knowledge of this relationship in the case of some of the obligate root-infecting pathogens is meager. There are, therefore, many possibilities of worth-while future study in this interesting group of plant disease organisms.

V. Prospect

In what has been written so far, an attempt has been made to cover in brief outline some of the basic facts and speculations relating to the interactions of pathogen, soil, other microorganisms in the soil, and host. The rapid progress made in recent years toward a better understanding of these interactions has ushered in many new ideas such as the concept of rhizosphere and rhizoplane microfloras and of vivotoxins. There is no doubt that future work will have to be largely molded by these two concepts which need to be fortified further by critical study. A newer approach to investigations on the rhizosphere is called for here which should take into account not only root exudations, and the impact of microbial metabolic products formed *in situ* in soils on root-infecting pathogens and the rhizosphere, but also the ionic balance or imbalance and osmotic changes within living and infected plant roots. In the present state of our knowledge, or ignorance, it would appear that the ionic status of many of the key elements in the living healthy and in diseased plants would have to be determined accurately, since chelation between heavy metals and products of host-pathogen interaction has been postulated. We do not yet know if such chelation is an invariable feature within living plant systems, but if it really is, then possibilities of such chelation mechanisms leading to essential heavy metal starvation in plants would have to be seriously reckoned with. After all, vivotoxin production and its complicity in disease would depend on the host substrate and we do need detailed information on these. It is to be hoped that at least some of these problems would be taken up in the not distant future and, with the extraordinary refinements in modern biophysical and biochemical techniques such as spectrochemical and chromatographic analysis, radioactive tracers and tissue culture, the horizon of our knowledge can be widened considerably.

Similarly, root-infecting obligate parasites also call for intensive study, since our understanding of the physiology of host-parasite-soil interaction in this group of pathogens is appallingly meager. What, for instance, is the difference between healthy and warted potato tissue? What is it that stimulates tumor formation in potato infected with wart disease, or tobacco plants suffering from virus clubroot? Or again, do normal healthy roots of cabbage differ in their exudates and rhizosphere microfloras from cabbage roots infected by *Plasmodiophora*? These are questions for which we have at present no answer. It is true that extensive studies have been carried out on the physiology of parasitism of leaf-infecting obligate parasites such as the powdery mildews and the rusts; but the root-infecting pathogens have been left behind. Studies of rhizosphere microfloras, microclimatology, root exudates, and of *in vivo* changes in ion accumulation, growth substance levels, permeability, osmotic changes, cellular oxidase systems, etc., will, therefore, have to be extended also to these root diseases caused by obligate parasites.

There is another fundamental problem of great interest that invites urgent attention. It has been suggested time and again that plant viruses, particularly the so-called soil-borne viruses, may have some relationship with soil fungi or other soil microorganisms for their multiplication. For instance, tobacco necrosis virus has been shown to survive in soils, but just how it does so remains unsolved, although the possibility has been visualized of soil microorganisms serving as hosts. It appears that this problem will have to be worked upon in considerable detail if we are to arrive at a solution. The idea in bringing root-infecting viruses into this discussion is to enlarge our experimental outlook for the future. In the event of viruses being shown to have intermediate hosts among microorganisms, the question would then arise as to how, when other than the angiospermous hosts, the gymnosperms, pteridophytes, and bryophytes have not been known to suffer from virus diseases, the microscopic members of the plant kingdom could be infected.

It may be conceded, no doubt, that, by the nature of the very material which requires study, experimental investigations on these problems are beset with many difficulties. Nevertheless, with the aid of suitable modern techniques it should be possible to take up these problems with confidence and imagination and study them vigorously and intensively.

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CHAPTER 9

Toxins

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I. THE TOXIN CONCEPT

The living plant must be regarded as a complicated biochemical factory carrying on a series of intricate and well organized processes essential for life. Any disturbance of these processes or the forces integrating them leads to a condition of "ill health" or disease. Recent studies on antibiotics have drawn attention to the high biological activity of many products of microbial fermentation. It is not surprising, therefore, to find that increasing attention is being focused on the role of toxic substances in plant disease. The difficulties of defining the boundaries of this discussion as distinct from a general discussion on the biochemistry of plant disease are obvious. The term "toxin" has been adopted in the plant pathological literature to describe a toxic substance rather than in the more restricted sense originally implied in the terminology of mammalian pathology. It is defined for purposes of this chapter as a product of a microorganism or a microorganism-host interaction which acts directly on living host protoplasts to influence either the course of disease development or symptom expression. The plant viruses have been deliberately excluded from consideration here. Enzymes, unless they are themselves toxic cannot be considered as toxins. Death of tissues in the soft rots for example (Wood, 1955) may result from cellular disorganization following the utilization of the materials of the middle lamella as an enzyme substrate.

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Gäumann (1954) states "Microorganisms are pathogenic only if they are toxigenic: in other words, the agents responsible for diseases can damage their hosts only if they form toxins—microbial poisons—that penetrate into the host tissues." This concept would seem, on the basis of current evidence, to be too embracive. In *Fusarium* wilt of tomato, for example, the organism enters the vascular system, often through root wounds and proliferates in the xylem vessels to which it is confined. Evidence, recently summarized by Dimond (1955), suggests that pectic enzymes are produced which, acting on the cell wall, hydrolyze or partially hydrolyze pectic materials with the resulting production of pectic gels and perhaps ultimately gums which plug the xylem vessels (Ludwig, 1952), thus interfering with water conduction and resulting in wilting and ultimate death. This situation does not involve toxins or any direct effect on living cells. The so-called wilt toxins (Dimond, 1955); (Gäumann, 1957), undoubtedly responsible for some of the commonly recognized symptoms of the disease, may play an entirely secondary role. They will be considered in more detail in a later section.

Dimond and Waggoner (1953b), concerned with distinguishing between phytotoxic products produced in culture media and those associated with disease in plants, coined the term "vivotoxin." They defined a "vivotoxin" as a substance produced in the infected host by the pathogen and/or its host, which is not itself the initial causal agent of disease. Influenced by Koch's "rules of proof" they propose three criteria for the establishment of a "vivotoxin," namely: (a) isolation from diseased but not from healthy plants, (b) chemical characterization, and (c) production of disease symptoms or a portion thereof on reintroduction into a healthy host. The only clear-cut examples of toxic materials that appear to meet all these requirements are ethylene, Dimond and

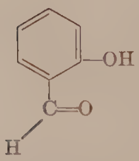
Waggoner's own example, and fusaric acid. This concept, now rather widely accepted in plant pathological literature, has served a useful purpose in helping to clarify our thinking with regard to toxins and plant disease. It would seem, however, to be too restrictive since it is difficult to apply to a nonspecific toxin such as that produced by *Helminthosporium sativum* (Ludwig, 1957) or *Rhizoctonia solani* (Kerr, 1956) where initial production may be external to the host and the effect one of preconditioning the host to the advance of the pathogen. The term vivotoxin might usefully be retained to describe a certain type of phytotoxin.

The chain of biochemical events occurring when a pathogen invades its host may work in favor of the host rather than the pathogen. Thus, Millerd and Scott (1955, 1956) found that an agent uncoupling respiration from its normally associated phosphorylations occurred in mildew-infected plants. This resulted in cell collapse and the liberation of phenolic substances. The latter appear to inhibit further mildew development and a "resistant" type of reaction is the result. Müller (1956) suggested that the term "Phytoalexin" be used to describe antibiotic principles present in hypersensitive host tissues. They cannot, however, be readily set apart from substances included under the general term toxin. The significance of antibiotic materials occurring normally in the host and conferring disease resistance is just beginning to be appreciated. Catechol (Link and Walker, 1933) responsible for resistance to onion smudge and 2,3-benzoxazolinone (Virtanen and Hietala, 1955) for snow mold in rye are good examples. Such materials are beyond the scope of this chapter.

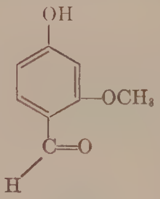
II. Toxins and Root Invasion

In 1918, Skinner published his classic monograph showing that aromatic aldehydes are produced in soil and might accumulate sufficiently under certain circumstances to be detrimental to crop growth. Among the more active of these aldehydes are salicylaldehyde (I) and vanillin (II). They are found especially in soils of low productivity and disappear rapidly from fertile soils having "strong biological activities and good oxidative powers." Poor fertility often favors root disease (Garrett, 1956). Rands and Dopp (1938) showed that apparently noninjurious amounts of salicylaldehyde predispose sugar cane to *Pythium* root rot. Similar observations (Graham and Greenberg, 1939) have been made in connection with *Pythium* root rot of wheat. Aldehydes of this type are among the first products of the microbial degradation of lignin (Brauns, 1952), although occurrence in nature may not be confined to this source as evidenced by the work of Gray and Bonner (1948a, b), and Bonner

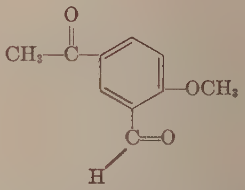
(1950) on the phytotoxic principle in *Encelia* leaves. This principle, shown to be 3-acetyl-6-methoxybenzaldehyde (III), inhibits growth of sensitive plants such as tomato at concentrations as low as 20 p.p.m. Fallen *Encelia* leaves retain their toxicity toward tomato for at least a year and the toxic factor can be leached into the soil by water. Finally, Schreiner and Shorey (see Skinner, 1918) isolated α-methylisonicotinic acid (IV) from infertile soil in the Tacoma district of Washington and found it to possess strong growth inhibitory properties.



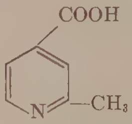
(I)
salicylaldehyde



(II)
vanillin



(III)
3-acetyl-6-methoxybenzaldehyde



(IV)
α-methyl isonicotinic acid

Ludwig *et al.* (1956) showed that artificial infestation of soil by spores of *Helminthosporium sativum* PK and B is ineffective in inducing disease development in barley seedlings. Whole culture organic inocula were highly potent but their activity could be reduced or eliminated by leaching with water. Heat sterilization sufficient to kill the organism failed to eliminate activity. They found further that the addition of sterilized inoculum to nonsterilized planting soil predisposes the barley seedlings to invasion by a variety of organisms including *Pythium* and *Fusarium* species. Seedlings treated with active culture filtrates or crude preparations of the toxin show stunting, chlorosis, loss of normal tropic responses, and death; symptoms characteristic of *Helminthosporium* seedling blight but no necrosis unless invaded by secondary microorganisms. Ludwig (1957) found the toxin to be nonspecific in relation to host, affecting wheat, oats, and barley equally. While strains of the fungus varied in their ability to produce toxin, and high toxin production was associated with high pathogenicity, some other factor of organism, perhaps a second toxin, appeared to be involved in disease development. The conclusion is reached that the toxin (or toxins) produced by *Helminthosporium sativum* predisposes barley seedlings to invasion by inducing premature senescence. This effect is illustrated in Fig. 1. In nature, the organism growing on infested seeds, crop debris, or senescing coleoptile sheaths would have ample opportunity to produce the toxin which could then affect actively growing crown tissue and predispose it to invasion. This seems to be parallel to the response described for aromatic aldehydes (Rands and Dopp, 1938, Graham and Greenberg, 1939). Preliminary work on the chemical nature of the *Helminthosporium* toxin suggests that it is an unsaturated aliphatic aldehyde with a molecular weight of about 220.

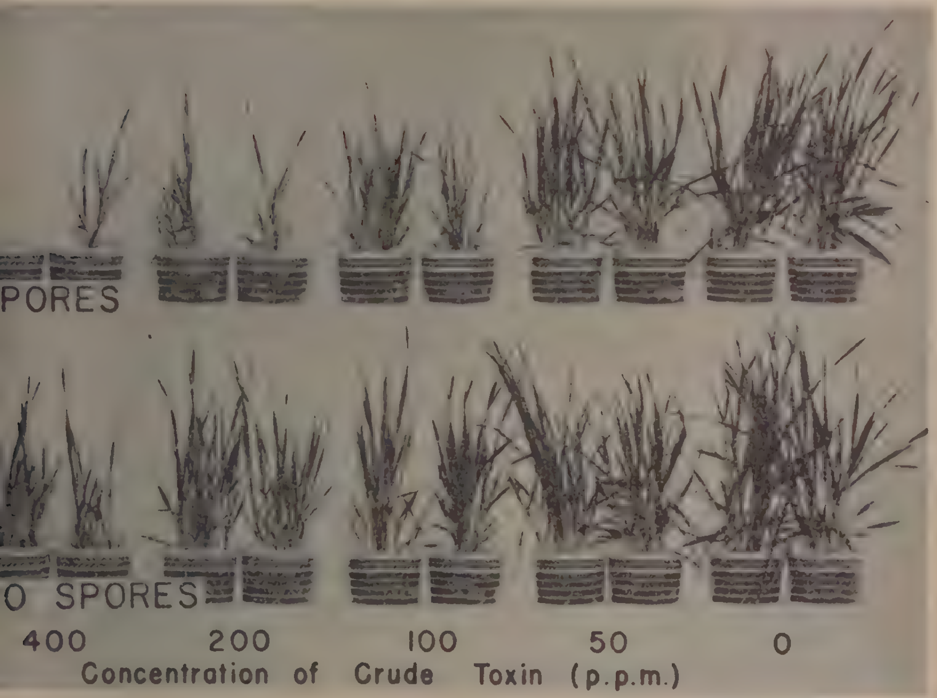


FIG. 1. The effect of treatment with toxin on the susceptibility of barley seedlings to *Helminthosporium sativum*. The plants in the lower row were watered once with the toxin solutions at time of emergence. Those in the upper row were treated with similar solutions to which spores had been added.

Helminthosporium oryzeae (*Ophiobolus miyabeanus*) causes a disease in rice similar to that produced in barley by *H. sativum.* Orsenigo (1956a, b; 1957) demonstrated the presence of a toxic substance in culture filtrates which reduced germinability and produced abnormalities in rice seedlings. He extracted the principle with chloroform and partially purified it by further solvent extraction. This product, cochliobolin, was found to be extremely toxic to rice seedlings, affecting root and shoot growth at low concentrations.

Kerr (1956) reported on infection studies with *Rhizoctonia solani* in which he employed a dialyzer tube to separate plant from inoculum. This barrier effectively prevented the passage of the organism but permitted the outward diffusion of chemicals from the root and the inward diffusion of products of microbial origin. *Rhizoctonia* was obviously affected by root excretions since its growth followed lines of root contact on the cellophane. Further, discoloration of roots occurred beneath the fungal mats, thus demonstrating a phytotoxic principle produced by the fungus. These results suggest that diffusing plant substances encourage the growth of the pathogen on the root where it evolves toxins which condition the host to invasion.

There is some evidence for toxin production by species of *Pythium.* Vanterpool (1933) found evidence of a toxin in his studies on *Pythium* root rot of wheat. More recently, Brandenberg (1950) studied the physiology of a species of *Pythium* found attacking the fine roots of beet. A toxic substance was found to be produced and it is postulated that it is swept from the root source to the foliage where symptoms are produced. The possibility of its being a pectic enzyme is not excluded.

Difficulty is often experienced in establishing a peach transplant in a site where an old tree has been removed. A number of workers (Proebsting and Gilmore, 1941; Upshall and Ruhnke, 1935) have shown that a toxic condition of the soil is associated with the "peach replant problem." Peach roots contain the cyanogenic glucoside amygdalin. Patrick (1953) demonstrated that both peach roots and pure amygdalin when subjected either to direct enzymatic or microbial breakdown produce substances toxic to peach seedlings. Among these degradation products are benzaldehyde and hydrogen cyanide.

Broadfoot and Cormack (1941) and Cormack (1948) studied a disease of alfalfa caused by an as yet unidentified low temperature Basidiomycete which they named descriptively "winter crown rot." Lebeau and Dickson (1955) demonstrated that the organism possessed the necessary enzymes for HCN production when an appropriate substrate was provided. Organic inocula, particularly those prepared from ground alfalfa crowns, promoted both kill and HCN accumulation in affected tissues.

Since HCN levels as high as 2400 p.p.m. were demonstrated in some tissues, they logically suggest that it is the agent responsible for death of cells. Winter snow and ice cover would block the outward diffusion of the gas that might be expected under other circumstances. The disease optimum of 2–4° C. is lower than the optimum of 12° C. for HCN production. The lower temperature might however be expected to favor accumulation (Bartholomew *et al.,* 1942). Here again then we would appear to have a situation in which the production of a toxin paves the way for tissue invasion by a microorganism. It is tempting to suggest in relation to the peach replant problem that microbially produced HCN affects the young tree and paves the way for root invasion perhaps by other normally nonpathogenic organisms, i.e., a root disease complex composed of two or more organisms none of which alone can establish themselves in the host.

III. Toxin Production by Alternaria

Toxin production has been associated with the development of early blight in Solanaceous hosts. Thomas (1940, 1948) observed chlorosis and necrosis in the top of tomato plants apparently only carrying the causal fungus, *Alternaria solani,* in a basal stem lesion. He also noted the rapid necrosis of leaf tissue around a point of infection. He concluded that these observations were most easily explained on the basis of a toxin produced by the fungus. Pound and Stahmann (1951) provided further positive evidence of the involvement of a toxin in the disease by studying the effect of various types of inoculation. Inoculation of the petiole on one side resulted in chlorosis or necrosis of the leaflets on that side. The fungus could readily be reisolated from the lesion but not from chlorotic or necrotic tissue away from it. A similar effect on the top of the plant resulted from one-sided lesions at the stem base. Here the leaves supplied by that portion of the vascular system under the lesion appeared to be the ones affected. Lesions on nonvascular leaf tissue had only a local effect.

Brian *et al.* (1949) isolated a biologically active substance from culture filtrates of *Alternaria solani.* They proposed the name "alternaric acid" for it. In later work, Grove (1952), showed it to be an unsaturated dibasic acid with an empirical formula of $C_{21}H_{30}O_8$. The details of its structure, however, yet remain to be elucidated. An apparently identical product was isolated by Pound and Stahmann (1951). Darpoux *et al.* (1950, 1952) described a biologically active material that they isolated from *A. solani* and gave it the name alternarin. It was inactive when sprayed on tomato plants but inhibited the growth of a number of fungi.

Alternaric acid possesses strong antifungal properties (Brian *et al.*, 1951). Spore germination in some species is prevented by 1 μg./ml. or less, while in others it is unaffected by 100 μg./ml. but growth of germ tubes is retarded soon after germination. Very low concentrations (0.01 μg./ml.) are required for the latter effect and, hence, this phenomenon was employed as a principle of bioassay in isolation studies with *Botrytis allii* as the test organism. Using this technique, production of alternaric acid or a biologically similar product was demonstrated in tomato fruits bearing *Alternaria* lesions, thus furnishing evidence of *in vivo* production.

Crude culture filtrates and alternaric acid itself are highly phytotoxic and when introduced into tomato shoots either through cut stem ends, petiole tips, or intact root systems, produce symptoms of chlorosis and necrosis characteristic of early blight (Pound and Stahmann, 1951; Brian *et al.*, 1952). It can, then, be carried upward in the vascular system. Alternaric acid is a relatively nonspecific toxin, although some plant species can withstand a somewhat higher level than others, and it can produce lesions resembling those of *Alternaria* in plants outside the host range of *A. solani.*

The extreme potency of alternaric acid, the striking similarity of its toxicity symptoms to natural infections, its translocatability and its known production on infected tomato fruits are all strong points of evidence in favor of its involvement in the early blight disease. Brian *et al.* (1952), however, found only 2 of the 12 strains of *A. solani* examined to be able to produce it in culture and these were the least pathogenic of those studied. This is not altogether surprising since *Alternaria solani,* in common with *Helminthosporium sativum* discussed in the previous section, tends to attack old or senescent tissues. Any factor tending to induce this condition whether it be age, adverse growing conditions, or the production of a nonspecific toxin would predispose the plant to attack. It would be interesting to know if unilateral petiole inoculations of all 12 of Brian's isolates would produce leaflet necrosis as demonstrated by Pound and Stahmann (1951) with their isolate. Finally, there is indirect evidence (Brian *et al.*, 1952; Darpoux *et al.*, 1952, 1953; Pound and Stahmann, 1951) that other toxins may also be involved. If this is the case it would further complicate the situation.

Black spot of Japanese pear caused by *Alternaria kikuchiana* is confined to a few varieties, all varieties of European pear and most of the Japanese varieties being either immune or highly resistant. Hiroe and Syoya (1954) found that sterile culture filtrates of the organism when applied to pear foliage induced symptoms identical with those of the disease itself. Pathogenesis of the organism and toxin production were correlated. These workers affected partial purification of the toxin and obtained some evidence of its production on infected fruit. The name "phyto-alternarin" was given to the toxin. It was shown to be different from alternaric acid. It is not clear whether or not the action of this toxin has the same host specificity as the organism.

IV. Diaporthin and Chestnut Blight

Chestnut blight caused by *Endothia parasitica* appeared in New York in 1904 and finally destroyed virtually all the chestnuts on the North American Continent. In 1953, Bazzigher showed that culture filtrates of the organism contained a toxin which he named "diaporthin." This substance was nonspecific in that it was shown to affect a variety of plants including chestnut and tomato. Further studies (Gäumann and Naef-Roth, 1957; Boller *et al.*, 1957) showed that at least two toxins are produced *in vitro* by *Endothia parasitica*. These are diaporthin with unknown chemical structure but empirical formula of $C_{13}H_{14}O_5$ and a bianthroquinone, "skyrin," with empirical formula $C_{30}H_{18}O_{10}$. The former is known to be produced only by *Endothia* while the latter synthesized by both *Endothia* and *Penicillium islandicum*. Both substances are relatively nonspecific in their action. As with many other toxins, the role of these materials in disease development has yet to be demonstrated.

V. Rice Blast

Blast, caused by *Piricularia oryzae*, is the most destructive disease of rice in Japan and other humid areas. The symptoms consist of spotting and severe necrosis on the leaves, culms, panicle branches, and floral structures. Tamari and Kaji (1954b, 1955) isolated two toxins from the culture filtrates of the organism. The first of these, "toxin A," with an empirical formula of $C_6H_5NO_2$ proved to be α-picolinic acid. The second, "toxin B," with an empirical formula of $C_{21}H_{18}N_2O_3$ yet remains to be identified. The authors applied the name piricularin to toxin B. Piricularin was found to be a much more potent growth inhibitor than α-picolinic acid, being active at a dilution of 1/400,000. At 1/1,000,000 it was stimulatory. Chlorogenic acid, stated to be the principal polyphenol of the plant, completely eliminated the growth inhibitory action of piricularin. The resistance and susceptibility of rice seedlings seemed to be related to their sensitivity to the combined toxins.

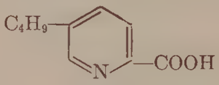
VI. Concerning Toxins and the Vascular Diseases

The so-called vascular diseases are caused by a group of pathogens capable of penetrating through the external tissue of the host and establishing themselves in the water-conducting tissue or xylem. Here they are usually confined to nonliving elements until the disease is in an advanced state of development, at which time they proliferate in the surrounding tissue. The fungi involved are a specialized group in that they can survive and multiply in this sheltered environment. They are generally nutritionally nonspecific and with the possible exception of the penetration phase of their life cycle lead a semisaprophytic existence and can be considered as showing a low order of parasitism, a characteristic in common with most of the fungi mentioned in the section on root diseases. By virtue of their location toxic substances produced can be either readily absorbed by surrounding cells or swept through the plant with the transpiration stream depending on their chemical nature and concentration. The most conspicuous symptom common to these diseases is a wilting and, hence, they are generally referred to as the wilts. Two theories have been advanced to account for the wilting. The first, the bundle plugging or occlusion theory assumes a vascular plugging interfering with water translocation and the second, the toxin theory, postulates plasma poisons which destroy the osmotic fuction of living cells particularly those of the leaf (Dimond, 1955).

A. *Fusaric Acid and the Fusarial Wilts*

The fusarial wilts, especially those of annual species have been extensively employed in studies on the physiology of hadromycotic wilting. Because of the many morphological and physiological similarities found in these diseases, some affinity among the toxins produced by their causal organisms would be expected if such toxins are indeed involved. This affinity appears to exist in their ability to produce fusaric acid.

Fusaric acid was originally isolated from *Fusarium heterosporium* Nees by Yabuta *et al.* (1934) and shown to be 5-butylpicolinic acid (V).



(V)
fusaric acid

Associated with it in some instances, for example in cultures of *F. oxysporum* f. *lycopersici,* is dehydrofusaric acid (5-butylenepicolinic acid). Its *in vitro* production by a number of fungi, all belonging to the family Hypocreaceae has been demonstrated (Gäumann, 1957). These include *Gibberella fujikuroi; Fusarium oxysporum* (f. *lycopersici,* f. *vasinfectum,* f. *niveum,* f. *batatos,* f. *nicotianae*); *F. solani, F. lateritium, F. monilformae, F. monilformae* f. *majus* and *Nectria cinnabarina* (Gäumann, 1957; Gäumann *et al.,* 1952b; Lakshminarayanan and Subramanian, 1955; Nishimura, 1957a). Undoubtedly, this range will be extended as more species and forms are examined.

Studies on the *in vitro* production of fusaric acid are often complicated by the presence of other toxic substances. The difficulty created by lycomarasmin production in the case of *Fusarium oxysporum* f. *lycopersici* was resolved by Kern (1952) who found that the fusaric acid inhibited spore germination in *Ustilago zeae* while the lycomarasmin was innocuous.

The nutritional conditions required for the production of fusaric acid appear to be relatively nonspecific, a C : N ratio of approximately 5 : 1 being more important than any specific feature of carbohydrate or nitrogen metabolism (Sanwal, 1956). Zinc has been found to be essential for both growth and fusaric acid production in *Fusarium oxysporum* f. *vasinfectum*. This is illustrated in Fig. 2, reproduced from Kalyanasundarem and Saraswathi-Devi (1955). It is particularly interesting to note that the critical range for fusaric acid production is much narrower than that required for growth. Fusaric acid production is not associated with autolysis. This is well illustrated in Fig. 3, adapted from Gäumann (1957). Dehydrofusaric acid, however, may well be an autolytic product. Kern (1952) compared lycomarasmin and fusaric acid production by a virulent and an avirulent strain of the tomato wilt fusarium and found the latter to produce slightly greater quantities of both substances, thus

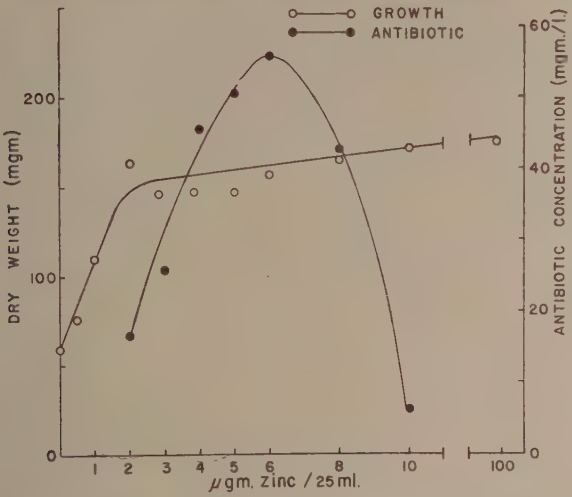


FIG. 2. The influence of zinc on growth and fusaric acid production in *Fusarium oxysporum* f. *vasinfectum*. (After Kalyanasundaram and Saraswathi-Devi, 1955.)

apparently confirming and extending the earlier observations of Gäumann *et al.* (1950) with respect to lycomarasmin. This might be interpreted as an argument against these substances playing an important role in disease development. The possible fallacy of such an argument was, however, demonstrated by Sanwal (1956) who found that this relationship could be reversed by a simple reduction in the nutrient concentration of the medium.

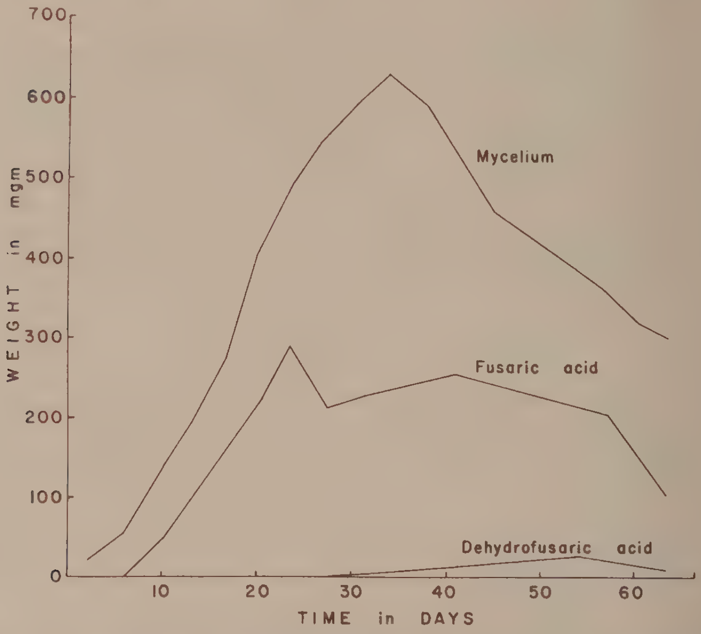


FIG. 3. Relationship between growth, fusaric acid production, and dehydrofusaric acid production in *Fusarium oxysporum* f. *lycopersici.* (After Gäumann, 1957.)

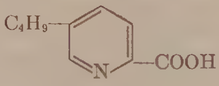
Lakshminarayanan and Subramanian (1955), and Kalyanasundaram and Venkata Ram (1956) using chromotographic techniques demonstrated the *in vivo* production of fusaric acid in cotton plants infected with *F. oxysporum* f. *vasinfectum.* Yields of at least 17 mg. per kilogram of fresh weight were obtained. Kern and Kluepfel (1956), using isotope methods, were able to demonstrate a fusaric acid-like compound in tomato plants 10 days after inoculation. Fusaric acid production in wilt-infected watermelon plants has also been demonstrated (Nishimura, 1957c). The probable production of fusaric acid within the plant suggested by *in vitro* studies has thus been confirmed *in vivo.*

The symptoms of fusaric acid injury to tomato are not typical of the symptoms observed in infected plants (Gäumann, 1957). Damage appears first in the stems where cortical tissue over the vascular bundles is destroyed resulting in a "furrowing." This gradually extends out into the petioles as injury progresses. Leaf injury, occurring later, usually takes the form of necrotic spots in the interveinal area. Epinasty follows stem injury. As would be anticipated, degree of injury and rate of development are functions of toxicant concentration.

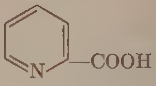
The treatment of tomato cuttings with fusaric acid results in an unfavorable disturbance of the transpiration-absorption balance (see Bachmann, 1956) and attempts have been made to relate this to changes in the water permeability of plant protoplasts. In the case of epidermal cells of *Rhoeo discolor*, a doubling of the rate of water movement was found to occur at a concentration of $1 \times 10^{-7}\,M$ (Bachmann, 1956). This effect decreased to a concentration of $1 \times 10^{-4}\,M$ after which a reduction in rate occurred. Comparisons of the cation content of diseased and healthy cotton shoots showed a substantial reduction of potassium accompanied by some increase in magnesium, calcium, and iron in the infected plants (Sadasivan and Kalyanasundaram, 1956). This is interpreted by the authors to mean a dearrangement of selective absorption and to be evidence against vascular occlusion as a primary cause of wilting.

Fusaric acid is a weak acid (Gäumann, 1957; Kern *et al.*, 1957) with percentage dissociations of approximately 2, 50, and 100 at pH's 4.3, 6.0, and 8.7, respectively. Cell penetration occurs most readily in the undissociated form and hence would be expected to increase as pH decreases. The practical application of this principle was demonstrated in an experiment in which tomato cuttings were placed in solutions at various pH's. Stem injury was found to decrease as the pH increased from 4.3 to 6.8 while leaf injury increased. Presumably, at low pH, the fusaric acid molecules diffused into cortical cells of the stem, whereas at high pH it was swept up to the leaves in the transpiration stream in ionic form. Gäumann (1957) points out that data on pH conditions prevailing within a tomato plant are not available but over the range given by Small (1955) for potato (xylem 4.0–4.4 to tubers, 6.2) the degree of dissociation would range from over 50% to virtually zero. Bachmann (1956), studying the effect on water permeability in cells of *Rhoeo discolor* and *Spirogyra* found pH to be only important at concentrations greater than $1 \times 10^{-5}\,M$. Below this level, permeation was pH independent. This finding may be a reflection of the cations readily available for chelation with the fusaric acid molecule. At low concentrations, cell penetration might be facilitated by metal complexing and the effect of pH on dissociation consequently masked. Once the supply of cations became exhausted, the degree of dissociation would then become important.

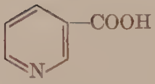
Deuel (1954) has recently reviewed the literature pertaining to the metal relationship of the wilt toxins. Fusaric acid (V) and picolinic acid (VI) are equally effective as growth inhibitors while nicotinic acid (VII) and the ethyl ester of fusaric acid (VIII) show little activity. The first two can form metal chelates while the latter two cannot.



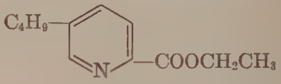
(V)
fusaric acid



(VI)
picolinic acid



(VII)
nicotinic acid



(VIII)
ethyl ester of fusaric acid

Bachmann (1956) found the ethyl ester to be as effective as fusaric acid itself in altering the water permeability of *Rhoeo* protoplasts. She concluded from this that metal chelation was not an important factor in activity. This discrepancy may be explainable on the basis of hydrolysis in certain biological systems.

Considerable emphasis has been placed by the Madras school on the metal chelating properties of fusaric acid in the physiology of wilt development. Lakshminarayanan (1955) found the activity of fusaric acid to be potentiated by iron. In analyses of resistant and susceptible plants cystine was demonstrated in the former but not in the latter (Kalyanasundaram and Saraswathi-Devi, 1955; Kalyanasundaram and Subra-Roa, 1957). Increasing temperature from 32.5° to 37.5° C. resulted in a progressive increase in detectable fusaric acid and decrease in wilt in infected plants. Cystine was present at 37.5° C. but not at 32° C. Preferential chelation of the iron by cystine, rendering it unavailable to the toxin, is suggested as an explanation of resistance. It has been demonstrated further (Subramanian, 1956) that pretreatment with 8-hydroxyquinoline protected cut shoots from the toxic effect of culture filtrates and functioned also as a chemotheropeutant on infected plants. Again preferential metal chelation is advanced as the explanation. While metal ions may influence the *in vivo* production and potentiation of the toxin, a poor chelator such as fusaric acid would not be expected to disturb trace elements present in a bound form and thus create deficiency symptoms. Finally the reaction of a susceptible cotton variety grown in infested soil was found to be altered to that of a resistant one by zinc fertilization (Kalyanasundaram, 1954).

B. *Other Toxins Produced by the Wilt Fusaria*

Lycomarasmin, isolated from cultures of *Fusarium oxysporum* f. *lycopersici* was the first of the so-called wilt toxins to be characterized. It is a dipeptide with a molecular weight of 277 and an empirical formula of $C_9H_{15}O_7N_3$ (Clauson-Kaas *et al.,* 1944). Woolley (1948) reviewed the chemical evidence and concluded that it is N-(α-(α-hydroxypropionic acid))-glycylasparagine. Lycomarasmin has been isolated only from culture filtrates of the tomato wilt *Fusarium* and apparently it either has not been recognized or does not occur in similar culture filtrates of other closely related wilt fusaria. Dimond and Waggoner (1953c), in their studies on the *in vitro* production of lycomarasmin concluded that it was a product of lysis rather than of actively growing mycelium. Gäumann (1957), however, takes exception to this conclusion and states that lycomarasmin can be demonstrated in the mycelium of *F. lycopersici* as early as the seventh day but reaches a maximum by the forty-fourth day. While *in vivo* production has not been demonstrated, the nutritional requirements for its production are relatively nonspecific (Dimond and Waggoner, 1953c) and its actual production within the conducting tissue of the living plants must certainly be considered a possibility.

Although lycomarasmin when applied to tomato cuttings does not produce symptoms typical of *Fusarium* wilt, this does not preclude the possibility that it plays a vital role in the development of the disease since a continuous low level of production within the host may have an effect quite different from a large single application applied to the base of a cutting. Lycomarasmin forms a chelate complex with iron that is far more toxic than lycomarasmin itself although it produces an identical symptom picture (Gäumann *et al.,* 1952a, b). The copper complex, by contrast, is nontoxic (Kern, 1956). Gäumann and Naef-Roth (1956) recently reported on the interaction between lycomarasmin, a weak chelator, and a series of cations chosen according to Mellor and Malley's (1947, 1948) stability series for coordinate bonding. "Komplexon III," a strong chelator was also included for purposes of comparison. Magnesium had no effect on either chelating agent. Manganese reduced the toxicity of the strong chelator but had little effect on lycomarasmin, a weak chelator. Cobalt, nickel, and copper detoxified both agents although increasing above a 1 : 1 *M* ratio resulted in metal ion toxicity. Iron is apparently carried to the leaves as the lycomarasmin complex. This then breaks down under the influence of light to liberate an excess of iron which is phytotoxic. When lycomarasmin is applied to the basal end of a tomato cutting, there may be a twofold effect: iron deficiency in lower regions resulting from the binding of iron and its translocation upward to the leaves and iron toxicity resulting from the release of an excess of iron in the leaf itself. Further evidence for this pattern of behavior was obtained by Gäumann and Bachmann (1957) who studied the effect of lycomarasmin on the protoplasts in cells of epidermal strippings of *Rhoeo discolor*. Lycomarasmin applied alone was found to have no effect on water permeability but when applied in a 1 : 1 *M* ratio with iron, produced a progressive increase in the rate starting with no effect at 10^{4} *M* to a doubling at 10^{2} *M*. This effect is illustrated in Fig. 4. It explains the fact that doses of lycomarasmin applied on successive days showed progressively less effect since available iron is swept away on the first day.

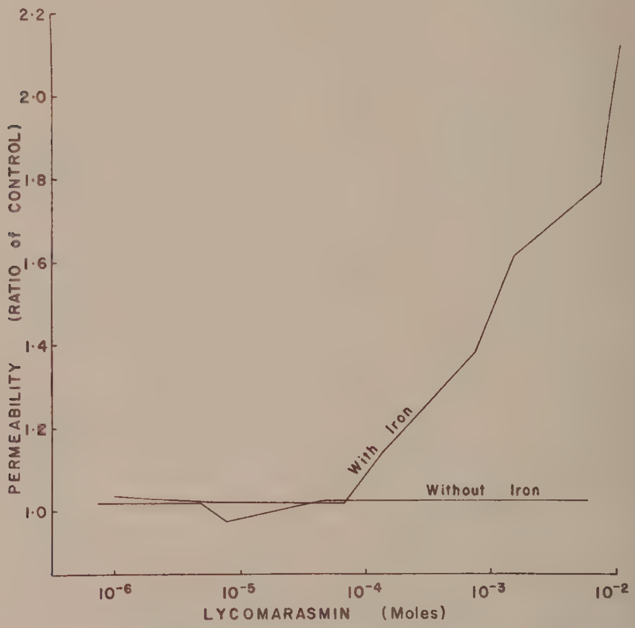


FIG. 4. The effect of lycomarasmin and its iron chelate on the water permeability of *Rhoeo* cells. The chelate was prepared by adding an equivalence of iron to each of the test solutions. (After Gäumann and Bachmann, 1957.)

Gäumann *et al.* (1952a) and Kern (1956) studied the effect of lycomarasmin on the water balance in tomato cuttings: "If tomato cuttings are allowed to take in the lycomarasmin-iron complex saturated with iron 1 : 1 they show the same characteristic curve of disturbed transpiration as they do with pure lycomorasmin although the saturated lycomarasmin-iron complex cannot chelate iron ions of the host on the way. The opposite occurs if lycomarasmin is shielded from iron so that on the one hand it carried no iron and on the other hand it cannot chelate on its way through the shoot iron ions of the host. In this case the transpiration of the tomato shoot does not show the characteristic curve of lycomarasmin injuries. Hence, lycomarasmin without iron is without effect in the field of excessive transpiration."

Hiroe and Nishimura (1956a, b) demonstrated the presence of a toxic principle in culture filtrates of the watermelon wilt *Fusarium* (*F. oxysporum* f. *niveum*) and succeeded in isolating it in crystalline form. They named the substance phytonivein. It was found to have an empirical formula of $C_{29}H_{46}O_2$ and to show many of the reactions of a steroid substance. Its occurrence in diseased watermelon plants was demonstrated chromatographically. There seemed to be a good correlation between *in vitro* production and the pathogenicity of strains of *Fusarium*. Cut shoots and seedlings of watermelon were permanently wilted by toxin concentrations of $10^{-5}\,M$. Phytonivein showed some chelating properties and appeared to affect the respiratory cycle (Nishimura 1956, 1957b, c).

Dimond and Waggoner (1953a) demonstrated the *in vitro* and *in vivo* production of ethylene by *Fusarium oxysporum* f. *lycopersici* and showed it to be the cause of the epinastic symptoms of tomato wilt.

Ethyl alcohol has been suggested as a possible cause of fusarial wilt induced epinasty in the tomato (Ludwig, 1952). Dimond and Waggoner (1953a) investigated this possibility and discounted it since amounts of ethanol greatly in excess of those demonstrable in either infected plants or artificial culture failed to produce the response. Scheffer and Walker (1954) inoculated plants of the resistant Jefferson variety and grew them in nutrient solutions containing 1.0, 0.5, and 0.2% ethyl alcohol. The treated plants were characteristically wilted in 12 to 15 days while the comparable uninoculated controls were apparently unaffected. No epinasty was reported in the latter. Resistance was unaffected in parallel experiments with methyl, isopropyl, and *n*-butyl alcohol. Alcohol concentrations as high as 2.1% have been reported for artificial cultures of *F. oxysporum* f. *lycopersici* (Luz, 1934).

C. *The Role of Toxins in Fusarium Induced Wilt*

Before discussing the possible role of toxins in the fusarial wilt diseases, brief reference must be made to two enzymatic processes. Ludwig (1952) advanced evidence to show that actual wilting is the result of occlusion of the conducting tissue. This conclusion was later strengthened by the findings of Scheffer and Walker (1953) who also showed that the active principle in culture filtrates is heat labile and nondialyzable indicating a substance or substances enzymatic in character. In subsequent work (Winstead and Walker, 1954; Waggoner and Dimond, 1955; Pierson *et al.*, 1955; Gothoskar *et al.*, 1953), pectic enzymes, primarily pectin methyl esterase, have been identified with this activity. The exact mode of action is not clear but presumably occluding plugs of pectic material form in the xylem vessels at the expense of the pectins in the cell wall (see reviews by Dimond, 1955; Wood, 1955). Evidence for this is to be found in the wilt-producing effects of enzyme preparations and histological evidence of pectic plugs and wall dissolution.

Vascular browning, another symptom characteristic of wilt, has also been ascribed to enzymatic action (Winstead and Walker, 1954; Davis and Dimond, 1954; Davis *et al.*, 1953; Gothoskar *et al.*, 1953). It appears to be due to the oxidation of phenolic substances to melanins by the polyphenoloxidases. The phenols involved may arise from the splitting of conjugated phenols, perhaps β-glucosides, by fungal enzymes. Gäumann *et al.* (1953) isolated a browning factor from culture filtrates. They called it vasinfuscarin, and suggested that it was enzymatic in character. Many preparations of the pectin esterases have also produced vascular browning. This is probably due to impurity rather than to the enzyme itself.

According to the concepts laid down in the introductory paragraph of this chapter, none of the enzymes involved here can be classed as toxins. The phenols could, however, be considered in this category since they exist in the stem of diseased but not healthy plants, and are directly responsible for the browning symptoms and may exert other physiological effects, perhaps even wilting (Davis and Dimond, 1954).

In fusarial wilt of tomato, a multiplicity of morphological symptoms precede and ultimately end in wilting and death. An attempt to reconcile all observations relating to the physiology of the pathogenism of the disease leads almost inevitably to the conclusion already published by Dimond (1955), namely, that what we observe is "the combined effect of many causes." Some symptoms such as epinasty, where ethylene is involved, can be traced to a single causal factor but this is the exception rather than the rule. Toxins are deeply involved as the previous discussion indicates and the work of Gottlieb (1943, 1944) on tracheal sap clearly demonstrates. Yellowing might be due to an effect of ethylene or to a robbing of iron from the chloryphyll molecule by lycomarasmin or an indirect effect accompanying a general senescence and decline of the plant.

The evidence implicating toxins as a primary cause of wilting is not strong. It consists first of the demonstration that a collapse in water economy occurs when cut tomato shoots are allowed to take up substances such as lycomarasmin, and second the demonstration that lycomarasmin and fusaric acid alter the water permeability of plant protoplasts. In the first instance, as already pointed out, the morphological symptoms produced by high dosages do not coincide with those associated with the disease. A collapse in water economy and wilting may be expected to result from the application of sufficiently large amounts of any translocatable nonspecific toxicant. Low dosages of lycomarasmin produce an increase in transpiration rate but no general breakdown. This certainly indicates a disturbance of physiological processes but is not convincing as the primary cause of wilting when the large changes in transpiration rate, occurring from day to day as a result of environmental changes, are considered. The same may be said for observed changes in the water permeability of the protoplasts where a doubling in rate has been recorded. The permeability of onion pulp cells, for example, has been reported as 0.3 ml. sq. cm. of cell surface per minute per atmosphere of difference in osmotic pressure (Davson and Danielli, 1952). The potential for water flow through a normal protoplast is, therefore, much greater than the actual flow. Irreversible increases in water and solute permeability are to be expected as death approaches and the osmotic system breaks down. It is easy to confuse cause and effect here and difficult to separate them experimentally. The recovery of detached wilted leaves and leaflets when placed in water (Dimond, 1955, and Ludwig, 1952) is evidence strongly in favor of an effect rather than a cause.

Biochemical comparisons of resistant and susceptible cotton plants have shown a number of physiological differences in addition to the differences in cystine content that have been correlated with wilt development. Resistant plants have also been shown to have a higher carbohydrate and ascorbic acid content than susceptible ones (Kalyanasundaram, 1955). Further, the pectin content of the roots of susceptible varieties has been found to be higher than that in resistant ones, synthesis and storage tending to be in the roots in the former and leaves in the latter (Lakshminarayanan, 1956). There appears, in other words, to be a correlation between root pectic reserves and resistance.

The ability of the organism to establish itself in the xylem vessels of a given plant appears to be governed by factors within the host (Kalyanasundaram, 1955; Gäumann, 1957; Scheffer and Walker, 1954) and is thus independent of the toxin-producing capabilities of the pathogen. During the early stages of the disease, in a susceptible variety, the fungus remains confined to the xylem and again is presumably excluded from the cortical tissue by the resistance factors of the host. Under cool conditions a plant may show little outward evidence of infection for a protracted period of time. At the onset of wilting, the organism grows out of the xylem vessels and proliferates rapidly in the surrounding living tissue. It is suggested that the nonspecific toxins produced by the organism pave the way for this final stage of the disease. Actual wilting may result from the combined effect of impaired osmotic function and the increased resistance to water movement resulting from vascular occlusion. The general association of fusaric acid with the fusarial wilts indicates that it may be the primary toxin concerned; further, the association of α-methyl isonicotinic acid with infertile soil (Skinner, 1918) and α-picolinic acid with rice blast (Tamari and Kaji, 1954b) suggests that a family of similar toxins may be involved in a broad group of diseases caused by weak parasites.

D. *The Verticillium Wilts*

Verticillium albo-atrum is an unusual pathogen in that it attacks a wide variety of plants including trees, shrubs, ornamentals, vegetables, and weeds with very little evidence of host specialization. Donandt (1932) for example isolated it from more than 70 plant genera and found each isolate to be transferable to each other host. Some differences in pathogenicity were noted but this would also be expected among a comparable number of isolates from a single host species. Bewley (1922), in his early studies on the sleepy disease of tomato, demonstrated that culture filtrates of the causal fungus could produce many of the symptoms of the disease itself when introduced into cut shoots. He concluded that the activity factor was enzymatic in character and that the wilting symptoms arise as a result of vascular plugging by pectin-like material. Others (see Green, 1954), have suggested nitrites as the wilt inducing toxin of *Verticillium albo-atrum*. More recently, Green (1954) reinvestigated the nitrite theory and found no evidence to substantiate it. The presence of wilt-inducing toxic substances in culture filtrates was reaffirmed. The fraction responsible for the responses, other than vascular browning, was found to be a proteinaceous material which did not appear in detectable amounts until after the onset of autolysis. A polysaccharide fraction also was isolated. This produced vascular discoloration and gummosis but only in the absence of the proteinaceous material. Green concluded that this discoloration is atypical and results simply from the inhibition of the amber color of the polysaccharide. In view of the association between activity and autolysis it is difficult to see any relationship here between *in vitro* and possible *in vivo* activity. Scheffer *et al.* (1956) found transpiration to be depressed before and during wilting and concluded that this is due to vascular blocking. Culture filtrates were found to contain a heat labile vascular browning factor.

The pectic enzymes produced differ quantitatively from those previously reported for *Fusarium oxysporum* f. *lycopersici* but the study of a broader range of material is necessary before any definite conclusion can be drawn in this regard. Talboys (1957) found some evidence that toxins are involved in *Verticillium* wilt of hops. These are, however, nonspecific since symptoms of desiccation and necrosis induced by culture filtrates bear no relationship to either the pathogenicity of the fungal strain or the wilt tolerance of the hop variety. The author suggests that there is a "determinative phase" in which the organism establishes itself in the vascular tissue and a later "expressive phase" in which visible symptoms appear and in which toxins are operative.

Young cotton shoots were found by Kamal and Wood (1956) to wilt rapidly when placed in culture filtrates of *Verticillium dahliae*. Toxicity of these filtrates to parenchyma cells appeared to be related to protopectinase activity while wilting seemed to result from the uptake of thermostable compounds of high molecular weight. Vascular browning was obtained only when solutions containing protopectinase were used. Here, however, only crude preparations were employed and hence the possibility of a contaminant cannot be excluded.

E. *Southern Bacterial Wilt*

Pseudomonas solanacearum attacks a wide range of solanaceous and other hosts. Hutchinson (1913) introduced the toxin theory of wilting as a result of his work on the Rangpuror Southern bacterial wilt of tobacco. His conclusions were drawn from the observation that an aqueous solution of an alcohol precipitate from beef broth cultures induces wilting when introduced into the tobacco plant. This view has recently been supported by Kunz (1952) who separated two toxic fractions from 2- to 5-month-old culture filtrates. One of these was thought to be a bacterial slime, complex polypeptide in nature, that occluded water conducting tissue and the second a plasma poison causing an osmotic disturbance in the leaves. Other workers (see Grieve, 1941) found no evidence of toxins and concluded that wilting is caused by a mechanical plugging of vessels by masses of bacteria. In an attempt to clarify this situation, Husain and Kelman (1958) made a detailed comparison of the behavior of strains of *Pseudomonas solanacearum* differing widely in their pathogenicity. Culture filtrates from young (24–48 hour) colonies of the virulent strain caused wilting in tomato cuttings while comparable filtrates from a weakly virulent and nonvirulent strain were without effect. Further, a heat stable viscous substance causing reversible wilting in tomato cuttings was isolated from filtrates of the virulent strain but not from the nonvirulent or avirulent type. This substance appeared to be a high molecular weight polysaccharide with glucose as its main component. Tracheal sap from plants inoculated with a virulent strain also induced wilting. This sap was shown to contain a polysaccharide similar to the one produced in culture. When grown on solid media, the cells of the pathogenic form were found to possess a slime sheath while the avirulent ones did not. This sheath appeared to be composed of the extracellular polysaccharide previously demonstrated. The evidence, to indicate that wilting results from a restriction of water flow caused by increased viscosity of the tracheal sap, is, thus, very convincing.

Polysaccharide production has also been demonstrated for a number of other plant pathogens and associated with wilting. These include *Ceratostomella ulmi* (Feldman *et al.*, 1950; Dimond, 1947), *Fusarium solani* f. *eumartii* (Thomas, 1949), *Verticillium albo-atrum* (Porter and Green, 1952), and various bacteria (Feder and Ark, 1951).

F. *Toxins and the Wilts of Woody Plants*

There are a number of wilts of woody plants that bear at least a superficial resemblance to the fusarial wilts in that their causal organisms are primarily vascular parasites and they are characterized by a yellowing and rapid wilting of foliage. Some progress has been made toward an understanding of the physiology of disease development despite the fact that the nature of the host makes experimentation much more difficult than with annual hosts.

Although frequent reference is made to toxins in the literature on Dutch elm disease, there is very little direct information concerning them. Zentmeyer (1942) freed culture filtrates of *Ceratostomella ulmi* of mycelium by passage through a Berkfield filter and injected them into young elm trees. After 3 days, typical symptoms of Dutch elm disease appeared. The principle involved was apparently nonenzymatic in nature since it withstood boiling for 5 minutes. Dimond (1947) obtained essentially similar results and showed that culture filtrates contain at least two toxic factors, one of which appeared to be a polysaccharide. Feldman *et al.* (1950) concluded that polysaccharides are not the main cause of the disease. They found the second toxic principle of Dimond to be unstable at high pH's. It accumulated in culture only at relatively low pH (4 to 5). In the field soil, applications of toxic chemicals were found to delay disease development. This could be an effect on toxin stability or simply an effect on growth. Beckman (1958) has shown that symptom expression in Dutch elm disease can be delayed by treatments that retard tree growth. There is then evidence of the production in culture filtrates of toxic substances which are circumstantially linked to disease development.

Oak wilt has been described as one of the most destructive tree diseases. While all oak species apear to be affected by the disease, it is most severe in trees of the red oak group. Symptoms progress from the crown of the tree downward. The leaves typically wilt, turn brown, and fall. The causal fungus, *Endoconidiophora fagacearum (Chalara quercina)*, is a typical vascular pathogen and appears to spread through a tree by movement in the transpiration stream (Young, 1949). Young (1949) found that oak cuttings, placed in cell-free culture filtrates of the organism, wilted rapidly and developed symptoms characteristic of those observed in small greenhouse inoculated plants. White (1955), in a more detailed study, found two nonvolatile thermostable toxins to be involved; one was responsible for the wilting and drying of the leaves while the other produced necrosis.

Nectria cinnabarina causes a disease of numerous trees and shrubs including apple and currant, frequently referred to as coral spot. This organism is a wound pathogen and invades the cortex and xylem causing a brown discoloration of tissues. The vessels become invaded by hyphae and their plugging by a brown gummy material is obvious. Invasion is limited and the mycelium of the fungus does not appear to extend much beyond the discolored area. The necrosis and wilting frequently noted in leaves above a diseased area have been variously attributed to a cutting off of water supply and to the formation of translocatable toxins (Uri, 1948; Kobel, 1951). Gaümann (1957) states that this organism also can produce fusaric acid. Kobel (1951) concludes that wilting, the major symptom, is due to an interference with translocation but that leaf spotting is due to the action of a toxin.

It must be concluded in relation to this group of diseases that toxins are definitely "suspect" but that a knowledge of their nature and role must await the outcome of future investigations.

VII. ETHYLENE AS A FUNGAL TOXIN

A study of substances which affect the physiological processes of the plant and in so doing may predispose them to disease leads almost inevitably to ethylene. This substance has been studied extensively by plant physiologists in relation to fruit ripening and is now generally accepted as the cause of the climacteric rise in the respiration of ripening fruits (Biale, 1950). It also induces fruit softening associated with pectic changes, notably an increase in soluble pectin and a corresponding decrease in insoluble protopectin. Ethylene has also been studied extensively in relation to auxin balance and foliar abcission. The literature relating to this question has been reviewed by Hall (1952). Ethylene promotes abscission but its effect can be counteracted by auxin treatment. Although both auxin and ethylene are less abundant in older tissues than they are in actively growing tissues it is the balance rather than the absolute amount that seems to be important in regulating the level of physiological activity in cells. Williamson (1950) confirmed the fact that a low level of ethylene production is normal in plant tissues and showed that it frequently appears in greatly increased amounts in response to injury or disease. He suggested that it might be responsible for rapid yellowing and early leaf abscission. It is a metabolic product of the green mold fungus *Penicillium digitatum* (Young *et al.*, 1951) and lemons infected with this organism show a respiratory rate as much as 100% greater than the controls.

Dimond and Waggoner (1953a) investigated the cause of epinasty, an early symptom of tomato fusarial wilt (Wellman, 1941). They found ethylene to be produced by the organism in culture and by diseased plants. The amounts demonstrated were adequate to cause epinasty and yellowing. Crude enzyme preparations from *Penicillium digitatum* and apple juice were found by Hall (1951) to produce ethylene from a variety of substrates, the best being ethyl alcohol, arabinose, and pectin. The observation with respect to ethyl alcohol is a surprising one and may, as the author suggests, indicate that it is the terminal substrate. *Fusarium oxysporum* f. *lycopersici* is known to produce ethanol both *in vitro* and *in vivo* (Scheffer and Walker, 1954; Luz, 1934; Dimond and Waggoner, 1953a). This could be of significance in relation to the appearance and role of ethylene in infected tomato plants. Pectin hydrolysis and ethylene production are not correlated since *Rhizopus nigricans* and *Penicillium italicum* failed to produce it although both are active pectin hydrolyzers.

Evidence summarized by Allen (1953) suggests that ethylene indirectly uncouples respiration from the energy-requiring activities of the cell.

All these results show that ethylene is biologically active in small amounts and that it seems to regulate the state of maturation of plant cells which in turn affects susceptibility to microbial attack. Studies on the role of ethylene in the physiology of a great variety of diseases would undoubtedly be very rewarding.

VIII. Wildfire and Angular Leaf Spot of Tobacco

Wildfire and angular leaf spot are two important bacterial diseases of tobacco. The former is characterized by localized chlorotic halos 1 to 2 cm. in diameter surrounding a central, usually small, necrotic spot while the latter appears as a small angular necrotic spot without the halo. The chlorotic area in the case of wildfire is free of bacteria. The causal organisms are *Pseudomonas tabaci* and *Pseudomonas angulatum*, respectively. Johnson and Murwin (1925) demonstrated that sterile culture filtrates of *P. tabaci* would produce wildfire symptoms. This observation was confirmed by Clayton (1934) who showed the toxic principle to be heat stable. Leaf prick inoculations with either the toxin alone or the toxin plus bacteria resulted in the development of typical wildfire symptoms on many plant species. Comparable inoculation with the bacteria alone, however, only gave consistent positive results on *Nicotiana* species. Thus, the toxin was shown to be nonspecific but its production dependent on the ability of the organism to multiply on the host concerned.

Braun (1937) made a detailed comparison of the two organisms, *P. tabaci* and *P. angulatum,* and found them to be morphologically, physiologically, and serologically indistinguishable. Strains could, however, be readily separated on the basis of host symptomology. Nontoxin-producing strains of *P. tabaci* were relatively easy to produce. These produced symptoms similar to those of *P. angulatum* on tobacco leaves. Since no evidence of the reversion of a nontoxin-producing strain to a toxin-producing one was obtained it is suggested that *P. angulatum* may be a strain of *P. tabaci.* Clayton (1936) described epidemic forms of the two diseases that are indistinguishable. Here the organism appears to spread so rapidly through water-soaked leaves that general necrosis precedes the development and diffusion of the toxin.

A tobacco leaf assay procedure has been developed for the wildfire toxin (Woolley *et al.,* 1952a) which was employed to monitor steps in isolation procedures and an apparently pure product was obtained. This product was highly active, 0.1 μg./ml. producing a halo on a tobacco leaf. It was also highly unstable, particularly at high pH's, and very hygroscopic. Analytical findings indicated the toxin to be an α-amino acid. This work was continued (Woolley *et al.,* 1952b, 1955) and the toxin found most probably to be the lactone of α-lactylamino-β-hydroxy-Σ-aminopimelic acid. Complete acid hydrolysis gives rise to the amino acid, α, Σ-diamino-β-hydroxypimelic acid, and lactic acid. The name tabtoxinine was applied to the former. It is inactive in either the formation or inhibition of wildfire halos. Treatment with dilute alkali results in an opening of the lactone ring.

Parallel to the work on characterization, studies on biological activity of crude toxin preparations were in progress. Braun (1950) found growth of the green alga *Chlorella vulgaris* to be inhibited by the wildfire toxin. Since this alga can be readily cultured, it lends itself to studies of this kind. The deleterious effect of toxin concentrations sufficient to inhibit growth completely was found to be completely overcome by the addition of liver extract to the medium. A large number of compounds, including those found in liver extract, were tested and of them only DL-methionine proved to be effective. The results indicated further that the effect was on methionine utilization rather than on its synthesis and that a constant ratio existed between the amount of toxin and the amount of methionine required as an inhibitor. Methionine is, in other words, synthesized by *Chlorella* in the presence of toxin but its normal utilization is impaired, i.e., competitive inhibition. Methionine sulfoximine, a known methionine antagonist, behaved in the same way as the wildfire toxin, with regard to both *Chlorella* and halo production on tobacco leaves. This work (Braun, 1955) was confirmed and extended with chemically pure toxin. The competitive reversal of the toxic action by methionine is well illustrated in Figure 5. This reversal could not, however, be demonstrated on tobacco leaves.

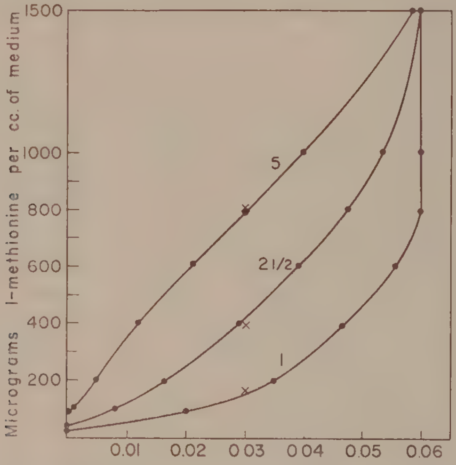


FIG. 5. Data obtained in an experiment in which three concentrations of chemically pure toxin were tested against a range of concentrations of L-methionine. *Chlorella vulgaris* was used as the test organism. Note that at half growth (X), an excellent competitive relationship exists between methionine and toxin concentrations. Five times as much L-methionine is required in a culture medium containing a 5-fold concentration of toxin as is needed to obtain the same amount of growth in a medium containing ⅕ that concentration. The results shown above were obtained after a 3-day incubation period at 25° C. (After Braun, 1955).

A number of structural analogues of methionine were examined for ability to produce halos on tobacco leaves and again methionine sulfoximine and the related ethionine sulfoximine were the only ones that proved to be effective.

Mutants of *Chlorella* selected for resistance to the methionine sulfoximine were equally resistant to the wildfire toxin. The evidence then suggests strongly that the wildfire toxin functions as an antimetabolite of the essential amino acid methionine.

IX. Bacterial Canker of Stone Fruits

The physiological relationship exemplified so well by the wildfire and angular leaf spot diseases may not be unique. *Pseudomonas mors-prunorum* Wormald is described as the organism causing bacterial canker and gummosis in stone fruits. This organism belongs to a group of organisms of the *Pseudomonas syringae* type apparently only separable on the basis of host range and pathogenicity (Erikson, 1945). Erikson (1945) and Erikson and Montgomery (1945) found that the organism invades the cells of the bark intercellularly causing plasmolysis of cells and later disintegration in advance of invasion. This paves the way for further advance of the organism. Cell-free filtrates, from old cultures of the organism grown on either a synthetic medium or bark decoctions, when injected into the bark of susceptible plum trees produce injury symptoms identical to those from an infection. Parallel filtrates from young cultures have little effect indicating that the active principle is a product of autolysis. A partially purified product was also found to be active. It is suggested that the killing action of *Pseudomonas mors-prunorum* may be due in part to an endotoxin of a proteinaceous nature. Varieties of cherry and plum resistant to the disease are little affected by the toxin preparations while susceptible varieties are severely damaged. Some strains of the closely related *Pseudomonas syringae* produce active filtrates while others do not. An understanding of the toxins produced by this complex group of microorganisms may well provide the answer to many of the problems posed by them.

X. Toxins and the Victoria Blight of Oats

In the preceding section, an example was given (*Pseudomonas mors-prunorum*) in which resistance and susceptibility appears to be regulated by sensitivity to a toxin. The most clear-cut illustration of toxin-regulated host specificity is, however, to be found in the case of the "Victoria" blight of oats.

The South American oat variety Victoria, an *Avena byzantina* type, shows a hypersensitive reaction to crown rust and beginning in the 1930's was used extensively in Canada and the United States as a source of resistance in the oat breeding program. Between 1944 and 1946 a new oat disease, confined to the Victoria progeny, appeared and increased to devastating proportions. It was characterized by a striping and yellowing of leaves, basal necrosis, and weakening of the lower culms resulting in severe lodging. The symptoms of the disease, its distribution, and the casual organism, a speciecs of *Helminthosporium* resembling *H. sativum* were described by Meehan and Murphy in 1946. They named the causal organism *Helminthosporium victoriae.* In a later paper (Meehan and Murphy, 1947) they produced evidence to show that a toxin is involved. The fungus is confined to the basal infection and cannot be isolated from blighted leaves till complete necrosis has occurred. Culture filtrates at high dilution produced leaf yellowing and streaking in the Victoria varieties characteristic of the disease but were without effect on resistant oat varieties. Finally, leaves of the Victoria varieties could be killed by spraying with culture filtrate while the resistant varieties were relatively unaffected. The principle in the culture filtrate was found to be heat stable since it withstood autoclaving for 20 minutes at 15 lb. pressure. These findings were later confirmed and extended by Litzenberger (1949) who demonstrated the presence of the specific toxic principle in infected plants as well as in culture filtrates. The fungus grew in the stems of both resistant and susceptible varieties when inoculated by needle puncture but only in the latter did symptoms develop.

Luke and Wheeler (1955) developed a chemically defined medium favorable to toxin production. Culture filtrates reduced root growth in susceptible types by 50% at dilutions of 1 : 1,000,000 but had virtually no effect on resistant varieties at 1 : 10. Further, a good correlation was found between pathogenicity, growth, and toxin production among strains of *H. victoriae.* The peak of toxin production coincided with the maximum growth. The toxin was found to be unstable at high pH but to be relatively stable at a pH of 4. This explains the success obtained with a medium containing ammonia as a nitrogen source, where the pH drops rapidly and remains at a low level until the ammonia is exhausted. Evidence of a second less active nonspecific toxin was obtained in this work. Unpublished results obtained by the author confirm this and suggest that the second toxin is similar to the one described for *Helminthosporium sativum.*

Wheeler and Luke (1955), through the use of the toxin, were successful in obtaining resistant plants from a susceptible variety by a mass selection process. Some of these seedlings were also resistant to crown rust.

Pringle and Braun (1957) isolated the toxic substance in what they consider to be pure form. They found that the toxin becomes less stable as purification proceeds and were unable, because of this instability, to obtain an elemental analysis, or to characterize structure. The product, however, was extremely potent, being active at 0.01 μg. per milliliter and produced the same host response as the crude culture filtrate and the disease itself. It appears to be polypeptide in nature. Fresh biologically active preparations of the toxin are ninhydrin negative (Pringle and Braun, 1958) but this reaction appears as activity is lost. Two ninhydrin spots appeared on paper chromatograms following mild alkaline hydrolysis. One of these was found to be produced by a compound having the empirical formula $C_{17}H_{29}NO$ and was given the name "victoxinine." The other appeared to be a peptide. Victoxinine completely inhibited root growth of both resistant and susceptible varieties at a concentration of 2.5×10^{4} *M*. Since it is nonspecific in its action, the authors suggest that the toxicity of the parent toxin may be due to the victoxinine while the specificity is a function of the peptide portion of the molecule.

The exact chemical nature and the mode of action of the Victoria blight toxin yet remain to be elucidated. It is one of the most intriguing of the phytotoxins demonstrated thus far, not only because of its extreme potency but also because of its host specificity. A final clarification of the problem may provide a clear-cut biochemical explanation of one type of disease resistance.

XI. Plant Growth Regulators

Plant growth is controlled by hormones and it is not unreasonable to assume that the effective level prevailing varies with species and variety and is genetically controlled. Went and Thimann (1949) for example demonstrated that growth in certain *Epilobium* hybrids is limited by an auxin deficiency and they were able to correct this by the application of auxin. Similarly, it has recently been demonstrated that dwarfism in certain varieties of corn and peas can be overcome by treatment with the gibberellins (Stowe and Yamaki, 1957). The question "can plant growth regulators sometimes be toxins?" is not an easy one to answer. It will be considered here in relation to the two main classes of plant growth hormones, namely, indole 3-acetic acid and the gibberellins. Much of the voluminous literature pertaining to this subject has been recently summarized in reviews by Brian (1957) and Stowe and Yamaki (1957). The latter review is particularly valuable since it summarizes Japanese work on the gibberellins, much of which is otherwise unavailable to Western readers.

A. *Indole Acetic Acid*

Indole 3-acetic acid (IAA) is a fairly common metabolic product of fungi and bacteria if tryptophan is supplied in the medium. Some fungi at least are also capable of producing on IAA-destroying enzyme. There are many plant diseases, particularly among those caused by obligate and semiobligate parasites, in which symptoms suggest an overdose of auxin. This observation has led to detailed studies, many of which have shown a high IAA content to exist. Crown gall is the best example. However, here, as pointed out by Brian (1957), there is no proof that the extra IAA in the tissues is of microbial origin. Further, there are two phases to tumor formation: (a) inception and (b) unregulated cell proliferation. The inception, or transformation of normal cells into tumor cells, apparently occurs under the stimulus of a tumor-inducing principle secreted by the bacterial cells. (see also recent reviews by Braun, 1954; Klein and Link, 1955). Similar demonstrations of a high IAA content have been made in connection with the so-called "witches'-broom" diseases, such as those caused by various rusts. Here again there is no evidence of *in vivo* IAA production by the microorganisms involved. Shaw and Samborski (1956) demonstrated that an area of high metabolic activity occurs in the vicinity of stem rust postules and mildew infections on wheat. These areas were high in IAA content even though no cell proliferation was induced. The evidence then would suggest that IAA is a result of enhanced metabolic activity rather than the cause of it. It is not, in other words, to be regarded as a toxin.

B. *The Gibberellins*

The temptation to regard the gibberellins as potential toxins is great as a result of the manner of their discovery. The bakanae or "foolish seedling" disease of rice is caused by a soil-borne fungus, *Gibberella fujikuroi (Fusarium moniliforme)* (see Stowe and Yamaki, 1957). Early attack by the organism results in a seedling blight. Tillering is suppressed in plants which survive the early attack of the organism and the remaining shoots as a result of excessive internode elongation grow taller than do those of the surrounding healthy plants. In humid areas, scab symptoms similar to those found in corn and wheat occur at heading time. The Japanese workers demonstrated that the symptoms of the bakanae disease could be reproduced by treatment with cell-free culture filtrates of *Gibberella fujikuroi.* Early work was made difficult by the presence of a growth-inhibiting factor in culture media. This complication was resolved with the identification of fusaric acid which has already been discussed. They eventually isolated a crystalline product that they called gibberellin A, (found to be a mixture of gibberellin A_1, $C_{19}H_{24}O_6$, and gibberellin A_2, $C_{19}H_{26}O_6$; see Grove *et al.*, 1958). Later, a second product, gibberellic acid, with the formula $C_{19}H_{22}O_6$ was isolated independently by British and American workers. Gibberellic acid and gibberellin A, although chemically distinct, appear to be physiologically identical.

Gibberellic acid reproduces the symptoms in the host characteristic of the bakanae disease and Brian (1957) concluded: "It is therefore virtually certain that the overgrowth in bakanae disease is caused by production of gibberellin A or gibberellic acid in the host plant tissue by the invading fungus." In the seedling blight stage of the disease, root growth is often inhibited while the coleoptile expands normally. These are symptoms of fusaric acid injury (Tamari and Kaji, 1954a). The gibberellins characteristically stimulate shoot elongation and are either without effect or stimulatory to roots (Stowe and Yamaki, 1957). It would seem, therefore, that both substances are operative in the disease, the nonspecific toxin, fusaric acid, tending to inhibit growth and perhaps predispose the plant to attack, and the gibberellins producing overgrowths in surviving less severely affected plants. The overgrowths characteristic of the bakanae disease are not caused by strains of *Fusarium moniliforme* other than those isolated from rice (Stowe and Yamaki, 1957) and parallel symptoms are not observed in such corresponding diseases as those of corn and wheat.

Following the demonstration of gibberellin production by *G. fujikuroi*, a number of workers have demonstrated the presence of gibberellin-like substances in higher plants (Radley, 1956). Recently, MacMillan and Suter (1958) have succeeded in isolating and characterizing gibberellin A from immature bean seeds, thus leaving no doubt that it is a new type of naturally occurring plant growth regulator and may be the forerunner of a series of hitherto unsuspected plant hormones. The gibberellins can, from this standpoint, scarcely be classed as toxins even though they are known to be responsible for one type of pathological overgrowth.

XII. Toxins and the Obligate Parasites

The toxin relationships involved in obligate parasitism must obviously be much more subtle than those prevailing in most of the examples considered in previous sections of this chapter. An ideal condition from the standpoint of the pathogen would be one creating the least pathological disturbance in the host. In the early stages of disease, substances produced by the organism might function as modifiers rendering the host more suitable for the further growth and development of the pathogen. The least favorable condition for the pathogen is the one involving a hypersensitive reaction where host cells die quickly, thus cutting off the development of the parasite. There is considerable circumstantial evidence for the association of host stimulating substances with rust and mildew infections on susceptible hosts. Yarwood and Jacobsen (1955) demonstrated that radiophosphorous accumulates around rust pustules on the leaves of Pinto bean. In one instance, one-half of a primary bean leaf was inoculated with rust. The shoot was then placed with the opposing, noninoculated, leaf in $H_3P^{32}O_4$ solution and the inoculated leaf in water. Under these conditions, 7870 times as much P^{32} accumulated in the rusted as in the nonrusted portion of the same leaf. In this experiment, as in many others, where the leaves were either immersed in water or in a saturated atmosphere, transpiration could not be a factor in accumulation. Further, accumulation was apparent shortly after infection and increased as the fungal development progressed. There was no sudden change in pattern as the developing pustule ruptured the cuticle. It might be argued that the accumulation represented uptake by the developing fungus rather than by host cells. A parallel increase was observed, however, with a number of mildew infections where the mycelium developing superficially could be removed, and with virus infections. Further evidence that the effect was one on host cells was provided by the fact that the accumulation of phosphorus also occurred after the rust had been destroyed by a selective heat treatment.

In experiments with rust and mildew on wheat, Shaw and Samborski (1956) found accumulation of C^{14}, P^{32}, and Ca^{45}. A number of sources of C^{14}, including sugars and organic acids such as indole acetic acid, were tested with essentially the same result. Among these, protocatechuic acid accumulated sufficiently to cause visible damage around infections while neighboring healthy areas were apparently unaffected. The curves for accumulation paralleled those for enhanced respiration. Either anaerobiosis or treatment with respiration inhibitors such as azide or dinitrophenol were effective in inhibiting accumulation. The results, in other words, suggest that the accumulation is similar to that observed in meristems and other metabolically active regions. The accumulation of indole acetic acid in the environs of an infection was not accompanied by increased cell division. This may, however, be a matter of species since Yarwood and Cohen (1951) did report some hypertrophy in connection with bean rust, a finding suggestive of the "tumor inducing principle" of crown gall (Braun, 1954).

Shaw and Samborski (1956), after a thorough discussion of their own and related findings, conclude: "The results obtained with *Puccinia* and *Erysiphe* are in full accord with the hypothesis that a diffusible substance (or substances), produced at the incipient infection, either by the fungus, or by the host cell or cells under attack or both, stimulates the metabolic activity of the host tissue in the environs of the infections." Such a substance would be a toxin. Its effect in the case of a susceptible reaction is to mobilize host reserves for the use of the pathogen. If, however, other translocatable phytotoxic substances are present, its effect might be to concentrate these at the infection site, thus causing death of cells and resulting in a hypersensitive reaction, i.e., an effect parallel to that reported for protocatechuic acid. While there is apparently no direct evidence for the latter role, the nature and time of production of such phytotoxins could account for the various intergrading rust reaction types.

"Victoria" oats, as has already been pointed out, are susceptible to attack by *Helminthosporium victoriae* but resistant to *Puccinia coronata*. The host selectivity of *Helminthosporium victoriae* is clearly governed by the reaction of the host to a specific toxin. Litzenberger (1949) showed that extracts from oat leaves infected with *P. coronata* possess a similar host specificity, indicating that the two unrelated pathogens may produce similar toxins. Damage to tissues in the case of the facultative parasite would lead to susceptibility while with the obligate parasite it would lead to resistance.

Allen (1953) reviewing the literature on "toxins and tissue respiration" notes that an enhanced respiration occurs in connection with a variety of plant diseases and that it is a response of the host cells to diffusible substances produced by the pathogen. He cites a limited amount of evidence to show that this results from an uncoupling of respiration from the energy-requiring processes of the cell through an effect on oxidative phosphorylations. This would not explain the rather prolonged period of enhanced metabolic activity associated with the growth of rusts and mildews on susceptible hosts. A complete or partial uncoupling could, however, easily be the explanation of a hypersensitive or partially resistant reaction. White and Baker (1954), in a histological study of barley mildew caused by *Erysiphe graminis* var. *hordei*, found resistance to be related to the rapidity and degree of collapse of the mesophyll cells. Infected leaves of a resistant variety (Millerd and Scott, 1956) showed a rapid rise in respiration followed by cell collapse at the infection site and return of the respiration rate to normal. The onset of increased respiration was delayed in a susceptible variety and the increase continued gradually as more cells became invaded. These results are illustrated in Fig. 6. The authors suggest that an uncoupling agent might be produced by the pathogen or host pathogen combination. Crude extracts from infected leaves (Millerd and Scott, 1956; Scott *et al.*, 1957) stimulated respiration in noninfected leaves. A phenolic compound, apparently liberated on cell collapse, was also isolated. It showed marked antifungal properties and was thought to prevent further development of the fungus. The "respiratory factor" caused cell collapse in leaves of a resistant but not a susceptible variety. Brushing the leaves of the susceptible variety with a preparation of the respiratory factor just prior to inoculation caused subsequent cell collapse and inhibition of growth of the pathogen. The hypersensitive reaction in barley can then, in at least one instance, be attributed to the action of a toxin and resistance and susceptibility seem to be related to the relative toxin tolerance of the barley varieties involved. The explanation of the Australian group, namely a partial or complete uncoupling of oxidative phosphorylation from respiration, could be the correct one. It seems more probable though that a stimulatory substance results in enhanced metabolic activity with resultant preferential accumulation of a phytotoxic substance. The latter might function as a respiratory poison.

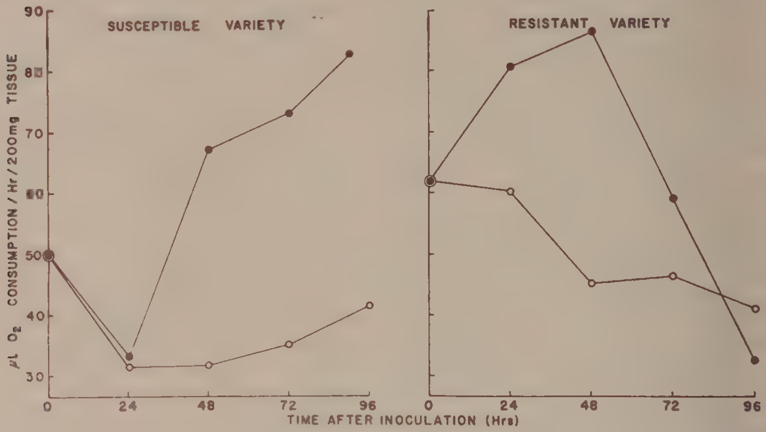


Fig. 6. Comparison of respiration of non-infected and infected barley leaves from a resistant and susceptible variety. (After Millerd and Scott, 1956.)

XIII. Summary

The importance of toxins in plant disease is becoming increasingly recognized and, although much of the information relating to their nature and role is still of a fragmentary character, certain generalizations are now perhaps possible. It is clear that toxins play a role in the physiology of pathogenism of all major groups of plant pathogens. Conclusions based on the criteria of symptom expression alone may, however, be misleading. In some instances such as the halo produced by the wildfire organism the symptom observed is the direct reflection of the action of a specific toxin. Localized necrosis as observed in many leaf spots, on the other hand, results from death of cells and can be caused by a variety of agents unrelated to the disease. Its exact form may be more a reflection of the particular host than of the pathogen involved. Similarly, changes in cell permeability or respiratory pattern may simply reflect impending death and as such be the effect rather than the cause of injury.

A variety of nonspecific toxins are involved in diseases induced by facultative parasites and facultative saprophytes. These toxins appear to act by predisposing the plant to invasion by the organism. A variety of physiological processes in the host are affected and the over-all effect can best be summarized by saying that they induce premature "senescence." In this, resistance factors of the host may be overcome or specific nutrients required by the microorganism may become available. The *in vivo* criterion is easy to apply in the case of a vascular disease where the pathogen inhabits the vascular system but is not applicable to a root pathogen initially located outside its host. Since senescence is involved, other factors, age for example, giving rise to this condition obviate the need for the toxin. It is not surprising, therefore, that only a very loose correlation can be found between apparent pathogenicity and the production of this type of toxin. Other factors of "organism" are involved since many saprophytes can produce nonspecific substances but are still unable to invade. Perhaps, as the studies on *Helminthosporium victoriae* would seem to indicate, a number of unsuspected host specific toxins are also involved.

There is good evidence for the association of a host stimulatory toxin (or toxins) with infection by obligate parasites. This may not be related to the host specific toxin demonstrated by the Australian workers in barley leaves infected with *Erysiphe graminis* var. *hordei.*

Finally, it is clear that a knowledge of the nature and mode of action of toxins is basic to a full understanding of the physiology of pathogenism. Such knowledge will ultimately lead to more efficient plant disease control through plant breeding and other means.

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CHAPTER 10

Heterokaryosis, Saltation, and Adaptation

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I. INTRODUCTION

Fungi have been described as "a mutable and treacherous tribe," but that even this is something of an understatement is abundantly evident from the frequent and spectacular outbreaks of fungus diseases in previously resistant plants. Variability in higher plants has long been recognized as a major principle, but its extent and importance have been fully appreciated rather late in the development of mycology and plant pathology. This appreciation has resulted largely from the failure of varieties bred for their resistance to a given pathogenic fungus to remain resistant when cultivated on a large scale. Furthermore, it is now realized that most fungi occur as specialized races differing in their ability to infect different plants. Such new races can arise as a result of variation in already existing races, and there is clearly a need for more information about the mechanisms responsible for these variations that determine the interactions between host and pathogen.

Since Mendel discovered the basic principles from which plant breeding has developed, many new varieties of host have appeared. This has meant that their specific pathogens have needed to change equally quickly and diversely, or otherwise not survive. If a particular host had no inherent capacity for variation in its reaction to disease, any pathogen would need only to appear in one form to ensure its survival. Variability within a species that acts as a host has, however, resulted in differing internal environments, each of which acts selectively upon the equally variable pathogens. Clearly, fungi which are inherently able to vary their pathogenicity to match each new situation as it arises can rapidly adjust themselves so that they retain parasitism. Variations on either side of the protracted battle between host and pathogen usually afford only temporary advantages for either participant, and although the plant breeder may produce disease-resistant varieties which resist attack by the existing physiologic races for a few years, the new varieties are likely sooner or later to succumb to the pathogen.

How do microorganisms make these adjustments in pathogenicity? It is now known that they can bring into play any one of a number of mechanisms that can result in genetic recombination, and the recently renewed interest in genetic variability in plant pathogens has revealed the presence of several methods by which this is very efficiently achieved.

The importance of variation in plant pathogenic fungi was first emphasized by Eriksson in Sweden (1894), who described different physiologic races of rust fungi. Shortly afterward, Ward (1903) and Salmon (1903, 1904) described changes in the pathogenicity of rusts and powdery mildews in England. Many of the major directions of approach and basic ideas on physiologic specialization were put forward at that time, but interpretations of these early observations are now open to doubt and explanations of variability in the light of more recently acquired genetic knowledge have become more generally acceptable.

Interest in this problem was renewed, some 15 years after Ward's experiments, by Stakman and his associates at St. Paul, Minnesota, who made a detailed analysis of the physiologic races of rust fungi and started a line of research that has since expanded enormously, and their results stimulated mycologists to look for similar evidence of race specialization among other fungi. Major contributions with soil-borne pathogens were first made by Stevens (1922) and by Christensen (1925), both working with *Helminthosporium*, and by Leonian (1929, 1932) with *Fusarium*. Brierley (1929) thoroughly surveyed the earlier theories on variability in fungi and advanced many new ones, particularly in the light of his work on variation in species of *Botrytis*.

In general, the variability in plant pathogens has been interpreted by four basic genetic mechanisms: (1) mutation, (2) heterokaryosis, (3) various recombination mechanisms, either with or without a sexual mechanism, (4) systems of adaptation, leading to changes in virulence and in tolerance to poisons.

Each of the four has been given prominence at different stages in the growth of the genetic outlook in plant pathology, and enough is now known to substantiate the claim that each can play an important role in the production and maintenance of variations. It is impossible to survey the whole of the literature on variability in pathogenicity, even in fungi alone, and this review attempts to assess the importance of variability by methods other than sex and gene mutation, both of which are dealt with in Chapter 11 of this volume.

Although much has been done, many questions still remain unanswered in studies of variation in almost any host-pathogen relationship. How, for example, do the imperfect fungi, which include many of the more important plant pathogens, manage to maintain such a remarkable ability to produce variant progeny despite their almost complete lack of a sexual stage? How important to the pathogen in nature are changes such as those that take place in the laboratory, and what are the most reliable ways in which the plant breeder can assess their importance? What is the extent of variation in any particular pathogen, and what guarantee is there that crops resistant in one locality will remain so in another, which may or may not harbor completely different physiologic races of the pathogen? Yet another urgent need is to formulate sensible and workable taxonomic systems of those fungi which vary so often and so widely morphologically. Before attempting to answer these questions, it is necessary to discuss briefly some of the problems involved in the concept of physiologic race specialization in plant pathogens.

II. Physiologic Races

One of the most important and obvious of the many ways in which microorganisms express variability is in the production of physiologic races. For the purposes of this review, those physiologic races that are distinguished from each other by their reactions on different varieties of a given host plant will be referred to as "pathogenic" races, because this maintains a clear distinction between them and the many other expressions of physiologic differences that can be separated by reference to other facets of microbial activity.

The pathogenic races of wheat rusts have been differentiated by inoculation to many different varieties of wheat, each race being able to cause disease on a given number of these varieties. Similar principles of race differentiation have been applied to many other plant pathogenic fungi, in particular to the smuts, powdery mildews, *Fusarium, Helminthosporium,* and *Phytophthora infestans.* Clearly the number of pathogenic races that can be recognized in any one plant pathogen by this method is a function of the number of differential hosts available for their separation. This situation raises the question of whether the races are already contained in the pathogen as separate genetic entities before the appropriate differential hosts exist, or whether the pathogen produces new races in response to the increasing appearance of resistant hosts. Is, in fact, differentiation into pathogenic races simply a matter of selection by host varieties of factors that have been present in the pathogen for a long time, or does the pathogen produce races almost as required? The supposition that the fungus produces races as and when new differential hosts are produced by plant breeders implies that they must possess genetic mechanisms that are well able to do this. If mutation were solely responsible for producing progeny that have increased virulence, and which are then selected by resistant differential hosts, a very high mutation rate would be needed to ensure that any newly formed race arrived on the most suitable host under optimum conditions for infection. However, bearing in mind the millions of spores that are produced by rusts, smuts, and *Phytophthora* spp., a mutational theory of the origin of new pathogenic races for these fungi is reasonable, but the same theory could not apply equally to many soil-borne pathogenic fungi, which not only produce few, if any, spores in nature but also have few, if any, opportunities for their dissemination to the new host varieties.

A no less likely theory than mutation would be that existing races might adapt so that they infect a previously resistant host, given the right conditions. With *Phytophthora infestans,* for example, the hypersensitive reaction of the leaves of a resistant potato variety toward invading germ tubes might well be prevented by the fungus producing an adaptive enzyme able to control a detoxification system, which could neutralize the inhibitory substances in the dead tissue of the blight lesion. Alternatively, in such examples where host resistance depends on a hypersensitive reaction to invasion by the pathogen, any decrease in the physiological activity of the fungus responsible for this reaction might also result in increasing the success of the fungus as a pathogen. If a change allowed it to avoid triggering-off the necrosis of the cell on which it depends for its nutrition, then it could proceed successfully to invade the rest of the leaf. However, answers to such problems as these must await the much needed research into mutation rates and adaptive potentialities of *Phytophthora* and of the many other fungi that also seem capable of extending their virulence and host range in parallel evolution with the ingenuity of the plant breeders.

As a result of their large-scale attempts to find the basic mechanism underlying the origin of races in *Puccinia graminis* var. *tritici,* Stakman and Piemeisel (1917) differentiated many pathogenic races of rusts on grasses and wheat and showed that each race could contain a number of biotypes, each having different degrees of virulence, yet still restricted to the one host range. This distinction between pathogenic race and differences in virulence within a pathogenic race should be kept clear when describing the changes in pathogenicity that are brought about by the different mechanisms of variation.

In these and many other examples of pathogenic specialization it is important in relation to disease control to know not only the exact host range, but also the geographical distribution and the factors influencing changes in the prevalence of races. Phenotypic variation of race expression is well known, as in the case of race 15B of wheat stem rust, which attacks some Kenya wheats at 65° F. but has no effect on them at 85° F. Similarly, low light intensity together with a low temperature increased host resistance to 10 races from a collection of 46 isolates of *P. triticina* in England and Wales (Roberts, 1936). Further environmental effects on races were found by Flor (1940), who classified 24 races of rust on flax and discovered that races with a limited range on flax varieties predominated in Midwestern United States, despite the fact that other races, which had a wider range, also existed there. One well known example of change in prevalence of a race of *P. graminis* var. *tritici* is afforded by race 15; this was first found in the United States in 1918 and it gradually increased, despite the introduction of resistant hosts, to a peak in 1938, since when it has gradually fallen off again. However, are environmental effects of this kind reflected by changes in host resistance, or by changes in the pathogenic races?

We also need the answers to many other specific questions. We still do not know how new races arise in nature, and whether the rate at which they arise during experimental handling in the laboratory bears any relationship to the rate in field experiments. How far can existing races give rise to new ones by sexual or asexual recombination systems, and what is the relative importance of these mechanisms to each other? The part played by mutation and adaptation is still relatively unknown. It is easy to overlook the fact that in many rusts and in wilt fungi, and in *Phytophthora infestans*, a great deal of pathogenic race differentiation goes on in the absence of the regular occurrence of a sexual stage. Despite the fact that suitable hosts for the development of sexual stages have been removed, as where barberry has been eradicated for rust control, or that sexual stages are virtually unknown in the most heavily cropped areas, as with *Phytophthora infestans*, or that they have never been found, as in *Fusarium oxysporum*, these fungi can still maintain and increase their heterogeneity.

The concept of the difference between one race and another needs continual revision, for we still have very little idea of what the exact differences are, beyond the bare fact that one race will attack a certain plant while another cannot. Resistance is usually inherited by the host plants as though it depended on a single gene, and the ability to infect seems to be equally simply inherited, at least in those fungi that lend themselves to genetic analysis. However, what exactly do the different genes for parasitism control? Or, to put it another way, what kind of resistance mechanisms do the races have to overcome? It seems feasible that some may depend on overcoming specific toxins in the tissues of a potential host, whereas others may need some nutrient that a resistant leaf or root lacks, but until more is known about resistance mechanisms in the differential hosts, our concept of races must remain empirical. Some attempts have been made to identify pathogenic races *in vitro* by serological and biochemical methods, but they have so far proved unsuccessful, or at best severely limited. Meanwhile, genetic studies of the inheritance of race type or of the basis of the origin of new races can be considerably furthered by using artificially induced genetic markers as extra aids in identifying progeny from crosses between different races. Indeed, by using markers for nutritional deficiency, it may at the same time be possible to pinpoint the nature of resistance of the different varieties to pathogenic races. Studies such as those described by Kline *et al.* (1957), Buxton (1956), and others, on the effect of adding the required nutrient to "deficient" races when inoculated to resistant hosts may well result in a fuller understanding of the yet unsatisfactory phrases "genes for resistance" and "genes for pathogenicity."

We are primarily concerned here with the changes that occur in pathogenic races and their biotypes in the absence of a sexual recombination cycle. Although gene mutation can account for some of the changes, particularly in examples where other mechanisms of variation are not readily demonstrable, new techniques and new approaches to fungus variation are revealing that many other ways are open for important changes to occur. Here we shall consider heterokaryosis, saltation, and adaptation as factors affecting fungus variability. Changes in pathogenicity will be held to concern changes both in virulence and in host range. Changes in host range imply an alteration in pathogenic race, and any change in the extent to which a particular race parasitizes a particular host is regarded as a change in virulence.

III. HETEROKARYOSIS AS A FACTOR IN VARIABILITY

A fungus is heterokaryotic when it contains two or more genetically different nuclei in a single hypha or spore. The nuclei may occur singly in each cell of the hypha or in multinucleate cells. With systems of this kind, variability is potentially large and different associations of nuclei can lead to a great variety of capabilities in a fungus. It is not, therefore, surprising that heterokaryosis has been invoked to explain many different expressions of variability. As early as 1929, Brierley, in an attempt to classify variability in fungi, described fungal "mixochimaeras," which were produced by fusions between hyphae. In an earlier essay he used the term "heterokaryotic" to describe mycelia containing cytoplasm and nuclei of different types, and he suggested that new strains of fungi might arise as a result of it. He also argued that the so-called "mutation theory" put forward to explain sectoring in fungi in culture was less likely than one based on the segregation of components from heterokaryons. Many of Brierley's ideas have since been vindicated.

A. *Cytology of Heterokaryons*

One important aspect of heterokaryosis, that of fungal cytology, has often been overlooked and it needs emphasizing at this stage of the discussion. The attention paid to the cytology of fungi which have been thought heterokaryotic has often been inadequate, and claims for this feature have sometimes been invalidated simply by showing later that the distribution of nuclei is not consistent with this supposition. Obviously, multinucleate spores could be heterokaryotic, so we need to know first how the nuclei of these spores originate. Detailed analyses of the movements of nuclei during spore formation have been made in many fungi, for example in *Helminthosporium sativum* (Hrushovetz, 1956), *Rhizoctonia solani* (Sanford and Skoropad, 1955), *Helminthosporium gramineum* (Graham, 1935), *Helminthosporium carbonum* (Roane, 1952), and in *Fusarium oxysporum* (Buxton, 1954). In *Fusarium*, both uninucleate microconidia and multinucleate macroconidia from the same mycelium were found to contain genetically identical nuclei, and during development of the macroconidia each nucleus arose by a process of division from a single original nucleus in the conidiophore.

In *Helminthosporium*, however, there is some evidence that the multinucleate spores could theoretically be heterokaryotic, for Graham showed that the several nuclei in a spore were not derived by division of one parent nucleus. By contrast, Roane's evidence that conidiophores in *H. carbonum* are uninucleate means that at least in that species heterokaryotic spores cannot arise. Hrushovetz's work adds a further complication, for he found that only one cell of the multinucleate spore of *H. sativum* usually germinated while the rest disintegrated, so that even supposing that the spores contained genetically different nuclei, they could not give rise to heterokaryons. Nevertheless, dispersal of genetically different monokaryotic spores as clumps would assure that heterokaryosis was perpetuated by the subsequent anastomoses between their germ tubes, and this kind of spore dispersal is in fact quite common in many fungi, as for example in certain fusaria and penicillia. To illustrate the diversity of the problem, examples of different kinds of spores and their cytological differences are shown in Fig. 1.

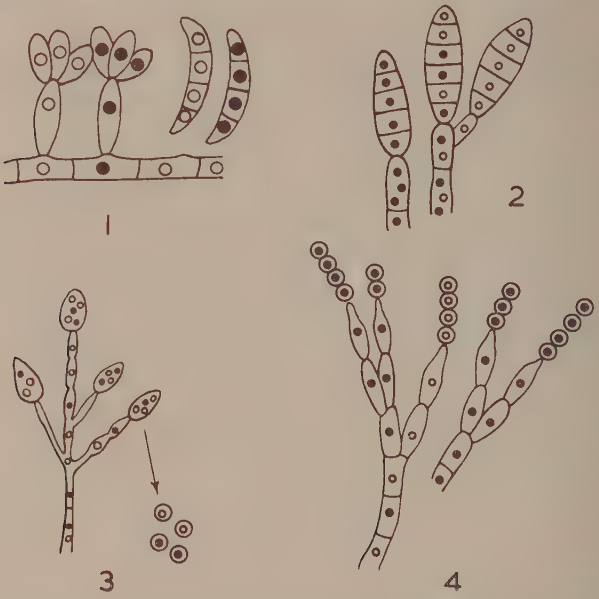
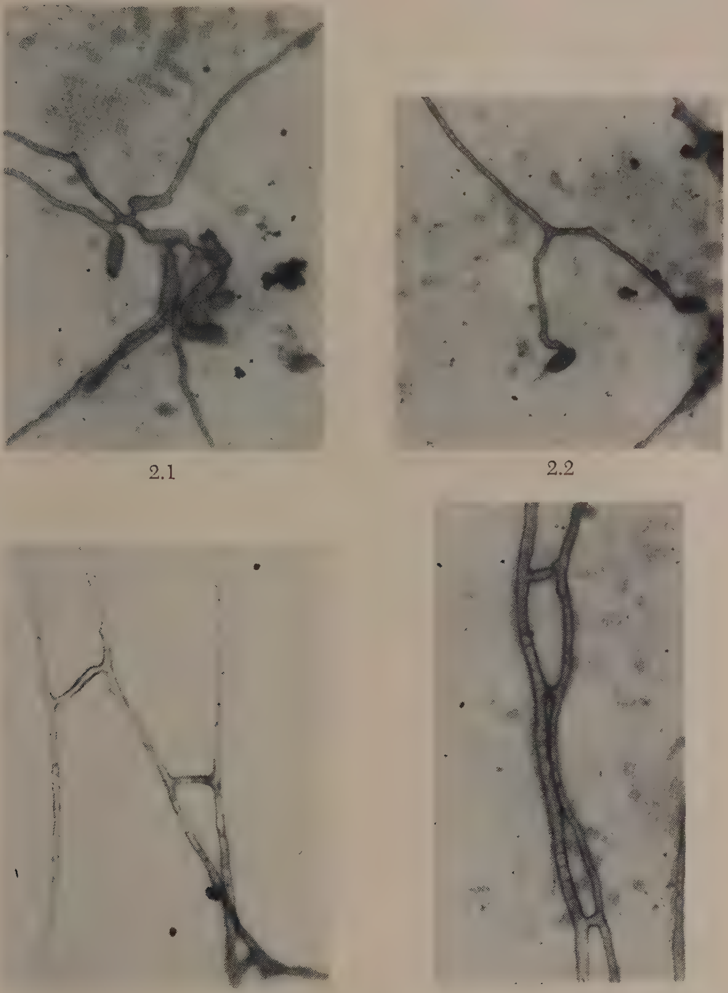


FIG. 1. Diagrammatic representation of nuclei in four fungi: (1) *Fusarium oxysporum*. Spores are homokaryotic; the mycelium can be heterokaryotic. (2) *Helminthosporium* spp. Spores may be homo- or heterokaryotic. (3) *Phytophthora infestans*. Heterokaryosis has not been demonstrated, but is theoretically possible. Zoospores are usually uninucleate. (4) *Penicillium* sp. Spores are homokaryotic, from a heterokaryotic mycelium.



2.1 2.2

2.3 2.4

FIG. 2. (1) Anastomoses between germ tubes from spores of *Fusarium oxysporum* f. *gladioli* (Buxton, 1954). (2) Anastomoses between germ tubes of two spores of *F. oxysporum* f. *gladioli* (Buxton, 1954). (3) Anastomoses between hyphae of two races of *F. oxysporum* f. *gladioli* (Buxton, 1954). (4) Anastomoses between hyphae of two races of *F. oxysporum* f. *pisi*.

Most heterokaryons probably originate as a result of anastomoses between germ tubes from spores of separate genetic origin, but they can also be synthesized at a later stage by anastomoses between adult hyphae (Fig. 2).

Nuclei migrate via the bridges between the anastomosing hyphae of the different homokaryotic haploid components. Although initiation of heterokaryosis is relatively easy to demonstrate, the mechanisms and changes in physiology underlying the way in which they are perpetuated is not yet clear. However, a fungus can maintain a state of heterokaryosis in a number of ways, as shown in Fig. 3.

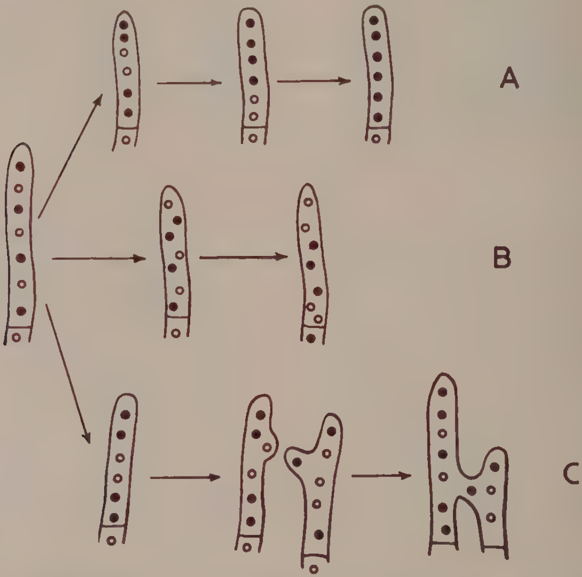


FIG. 3. Possible ways in which hyphal tips of heterokaryons may grow: either A, as homokaryons, or B and C, as heterokaryons (Buxton, 1954).

If a terminal nucleus continually divided and its progeny could not mingle with other dividing nuclei, a homokaryotic hyphal tip would result (A), but in many fungi the nuclei are small enough to allow movement past each other in the tips of the hyphae. Nuclear migration is well known in several fungi and it could easily result in a nonlinear arrangement of nuclei in hyphal tips. For example, the movements of nuclei in *Gelasinospora* (Buller, 1931; Dowding and Buller, 1940) have been fully investigated, and Sanford and Skoropad (1955) have observed nuclei migrating from cell to cell in *Rhizoctonia solani*. It appears then that the heterokaryotic condition can be relatively easily maintained in the hyphal tips, and it is significant that both in *Fusarium* (Buxton, 1954) and in *Penicillium* (Rees and Jinks, 1952) no cross-walls are formed near the tips, thus leaving a multinucleate terminal cell.

B. *Factors Affecting Anastomosis and Heterokaryotic Growth*

Among the many serious gaps in our knowledge of heterokaryosis, one of the most important concerns the optimal conditions for anastomosis between germ tubes or between older hyphae. So far only a few thorough studies of this problem have been made, although many workers have noticed in passing that anastomosis can be considerably affected by changes in the cultural environment. Hyphal fusions between strains of *Corticium vellereum* occur more often on Difco-Bacto potato dextrose agar medium at 25° C. than under several other cultural conditions (Bourchier, 1957); they are unaffected by changes in pH of the culture medium. Although Bourchier showed that adding sugars to the culture medium increased the number of fusions, Cabral (1951) found that an unsupplemented agar medium provided optimum conditions for fusions in various members of the Polyporaceae. These isolated examples of critical examination of the factors involved do at least indicate that successful anastomosis depends to some extent on the nutritional status of the culture, but there is recent evidence that genetic factors are also involved, not only in anastomosis but also in the ability to continue growth as a heterokaryon (Garnjobst, 1953, 1955). In *Neurospora crassa*, heterokaryons could be easily synthesized between two strains only when the genes controlling this ability were nonallelic. For example, an analysis of crosses between two isolates of the inositol-less strain 37401 revealed the presence of two nonlinked genes for heterokaryosis, which were designated C and D. Of the four possible classes of progeny in 37401, CD, cD, Cd, and cd, only CD genotypes were able to form stable heterokaryons with the riboflavinless tester strain Y 30539. In addition, this genetic incompatibility was unrelated to the type of nutritional deficiency in the tester strains. Fungi which have such a system of control over heterokaryosis obviously decrease the opportunities for their taxonomically related strains to make frequent union. It is probable that the occasionally reported failures to establish heterokaryons may be caused by the presence of similar genetically controlled incompatibility systems in other fungi. Leonian (1930), for example, was unable to make what he called "mixochimaeras" between all but four of several mixtures of spores from two morphologically distinct colonies which arose from a single isolate of *Fusarium moniliforme*.

C. *The Effects of Heterokaryosis on the Physiology of Fungi*

It is self-evident that the existence of two or more genetically different types of haploid nuclei within a hypha of a fungus gives it a wide range of genetic capabilities. This means that the combined action of the different genes in different nuclei can result in the fungus having a wider range of physiological activity than either of its original homokaryotic components; there are many examples of this phenomenon, which may appropriately be called "hybrid vigor," among heterokaryotic fungi.

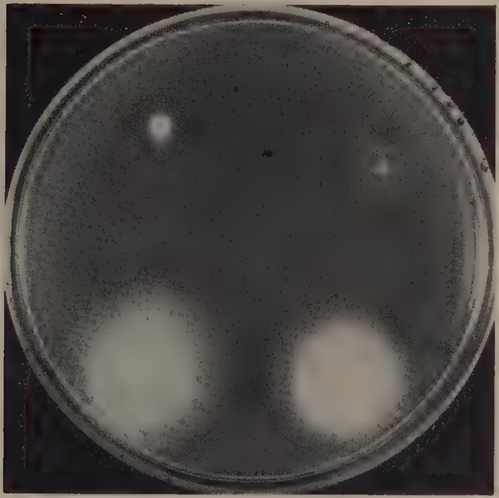
1. *Heterokaryosis and Cultural Metabolism*

The fact that heterokaryons usually both appear and act differently from their original component strains can best be demonstrated by using artificially induced mutants that cannot synthesize all their growth requirements. Take a simple example in which a mutant strain cannot synthesize an essential metabolite A, but can synthesize B, while another mutant strain can synthesize A but not B; the heterokaryon formed from them would contain genes, in separate haploid nuclei, which control the synthesis of both metabolites. The two kinds of nuclei each supply the normal allele of the mutant gene of the other, so that the heterokaryon, having a full complement of alleles, is no longer deficient and grows like the wild type. An example of such complementary behavior between nuclei of nutritionally deficient mutants of *Fusarium oxysporum* is shown in Fig. 4.

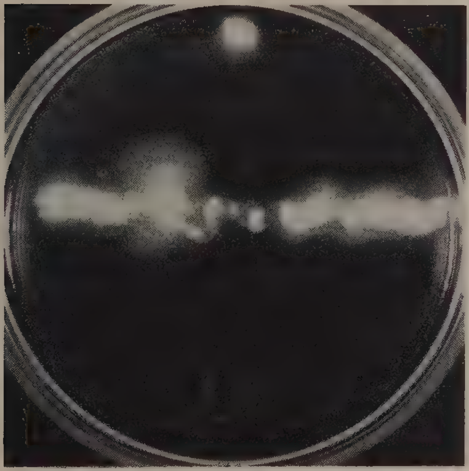
The increased range of physiological activity in heterokaryons could be significant for the survival of fungi under natural conditions, because a heterokaryon can colonize substrates on which its separate components might be unable to grow. Such a situation would arise when metabolites essential to either component are absent or in limiting concentration in the natural substrate.

In a related subject, the physiology of spore production has been shown to depend less on cultural conditions than on the segregation of genetic factors from heterokaryons. From wild type isolates of *Cercospora musae*, the cause of the serious "sigatoka" leaf spot disease of banana, Calpouzos (1954) was able to select heavily sporulating variants, whereas the majority of cultures sporulated only rarely or poorly. From cultures raised from single multicellular spores, he obtained both sporing and nonsporing strains, showing that the fungus probably occurred in nature as a heterokaryon.

Heterokaryosis in the wild type has also been recorded in *Penicillium*, in which the uninucleate spores are held together at dispersal, resulting



2.1



2.2

FIG. 4. (1) Top left: deficient mutant C25; top right: deficient mutant K27; bottom left: heterokaryon C25 + K27; bottom right: wild type parent of C25 and K27. *F. oxysporum* f. *pisi* race 1, growing on a nonsupplemented minimal medium. (2) Heterokaryon growing from a mixed spore streak of mutants C25 (top) and K27 (bottom). *F. oxysporum* f. *pisi* race 1, growing on a nonsupplemented minimal medium.

in heterogeneous groups of spores which grow in culture as reconstituted heterokaryons (Jinks, 1952a, b). Heterokaryotic colonies originating in this way, by anastomosis between germ tubes, can be picked up from exposed dishes of agar medium as single point isolates.

Not only does the possession of genetically different nuclei increase the physiological range of a fungus, but changes in the ratios between the numbers of each type of nucleus, in response to changes in the environment, can have important effects on its metabolism. Changes of ratio in response to changes in the nutrient status of the agar media can be assessed, with obvious reservations, by analyzing the types of colonies which result from plating out the spores produced by a heterokaryon. This has been done with *Penicillium* (Jinks, 1952b) and with *Fusarium* (Buxton, 1954). When a *Penicillium* heterokaryon containing two different kinds of nuclei was grown on a medium containing 10% apple mush, the initial ratio between the nuclei was 1 : 11, but it changed to 1 : 6 when the apple content of the medium was reduced to 2%. With *Fusarium*, increasing the carbohydrate content of the medium resulted in an increase of the proportion of one nuclear type over another from an initial ratio of 1 : 1 to a ratio as high as 4 : 1 (Table I). Various ratios, from 3 : 1 to 1 : 3, of the two nuclei also became progressively adjusted as the medium was changed, either in carbohydrate content or in its carbon : nitrogen ratio.

TABLE I

EFFECT OF GROWING HETEROKARYON F + A ON NUCLEAR RATIO OF F : A TYPES IN SPORES OF THE HETEROKARYON[a]

Medium to which hyphal tip transferred	Morphological appearance of heterokaryon	Types of single-spore colonies from cultural heterokaryons		
		Total colonies		Ratio F : A
		F	A	
CO/N_2	Adpressed, white	343	479	0.71
C_1/N_2	Slight aerial growth, white	158	499	0.33
C_2/N_2	More aerial growth, very pale buff	21	182	0.11
C_3/N_2	Increased aerial growth, pale buff	132	464	0.28

[a] Buxton (1954).

The flexibility resulting from such changes in nuclear ratios can rapidly adjust heterokaryons to a new food supply, with the advantages that the adjustments are made relatively simply in the somatic stage of the fungus, without the hazards and delays incurred by reliance on a sexual system. The advantages of such a mechanism are clear in the soil-borne penicillia and fusaria, in both of which adjustments of nuclear ratios presumably occur in the multinucleate hyphal tip cells.

From these examples of the effects of heterokaryosis, it is clear that this system of interaction between different haploid nuclei within one fungal thallus could be of great significance not only in fungus physiology but also in pathogenicity, and recent work has indicated that this is so.

2. *The Effect of Heterokaryosis on Pathogenicity*

To what extent can heterokaryosis influence the virulence and host range of a fungus? In other words, are there any synergistic effects between nuclei that can result in increases, even though only temporary ones, of virulence? If so, how often can such associations occur in nature, and under what conditions?

Like many other fungi, different isolates of pathogenic strains of *Fusarium oxysporum* can readily form heterokaryons when their spores are sown mixed together on agar media. Heterokaryosis is proved by subculturing hyphal tips from the colonies of mixed spore origin, for hyphal tips that are heterokaryotic produce colonies with spores which themselves will later form separate colonies of both the original component strains. The effect of heterokaryosis on virulence has been examined in *Fusarium oxysporum* f. *pisi*, the cause of pea wilt. Several thousand conidia from two isolates of the fungus, both of which wilted pea variety Onward, were irradiated with ultraviolet light and the resulting mutants were tested for virulence. The majority remained as virulent as the original strains, but several were much less virulent. When heterokaryons were made between pairs of nearly avirulent cultures and tested on peas, they were found to be as virulent as the wild types (Fig. 5). The heterokaryons were recovered by culturing from diseased vascular tracts of the inoculated plants, so demonstrating that the increased virulence had come from the heterokaryons and not from the possible synergistic effects of any segregant strains that might have arisen (Buxton, 1954, 1956).

From this evidence it is not unreasonable to suppose that some of the increases in virulence that occur in nature and lead to outbreaks of disease, may be the result of the chance union of two or possibly more weakly virulent strains as a heterokaryon. Any increased virulence resulting from heterokaryosis would persist only in mycelia and would not be inherited by spores; but sporulation, at least in *Fusarium*, does not usually occur until after damage to the host is complete.

Work by Hrushovetz (1957) suggests that a host plant may present a selective environment to individual nuclei contained in a heterokaryon if the nuclei determine different nutritional abilities or differences in virulence. He took several isolates of the heterokaryotic fungus *Helminthosporium sativum* and grew them in dishes of Czapek Dox agar medium each containing 0.1% of amino acid. After six successive transfers of each isolate on these media, which included either alanine, arginine, histidine, isoleucine, leucine, methionine, serine, threonine, or tryptophane, he found that their virulence toward wheat seedlings had been considerably altered. To explain this, Hrushovetz suggested that the amino acid supplementation effectively selected different nuclei from the heterokaryotic strains, resulting in the predominance of those strains requiring a particular amino acid for growth. He argued that, if the particular amino acid required by a strain were present in the host tissue, then that strain would develop, but would be relatively nonpathogenic. On the other hand, absence of an amino acid would result in the predominance of pathogenic nuclei in the heterokaryon. This kind of attenuation, depending on the selective activity of amino acids, was referred to as "apparent attenuation," for the acids apparently did not act as mutagens, but as selectors operating on an already differentiated genetic pool in the fungus.

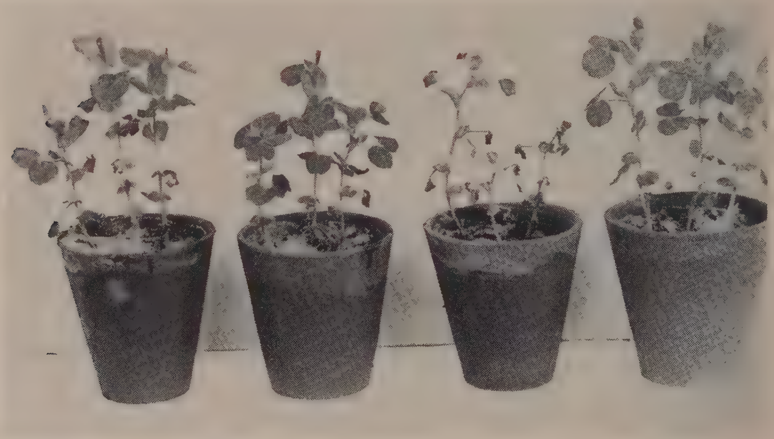


FIG. 5. Increased virulence caused by heterokaryosis in *F. oxysporum* f. *pisi* race 1, on peas variety Onward. First left: avirulent strain C25. Second left: avirulent strain K27. Third from left: highly virulent heterokaryon formed from C25 and K27. Right: uninoculated control.

The well-known variability in the pathogenicity of wheat rusts has been recently reexamined for evidence of heterokaryosis and for other mechanisms that may result in the production of new biotypes or new races, and, as in *Fusarium*, heterokaryosis is an important factor in altering their virulence. Wilcoxson *et al.* (1957) showed that germ tubes from uredospores anastomosed with each other when growing on the surface of wheat leaves. This gives opportunity for development of heterokaryons. Nelson (1956) took uredospores from two biotypes of different races of *Puccinia graminis* var. *tritici*, mixed them, and inoculated susceptible and resistant varieties of wheat with the mixture. In one experiment he isolated an orange-colored race from wheat inoculated with a mixture of a red-brown-colored race 11 and a gray-brown-colored race 121. The new orange race was highly virulent toward the wheat, Vernal Emmer, hitherto resistant to race 11. Not only did some of these new biotypes which arose from the mixture have three nuclei, but after six uredial generations they segregated back to the two original parentals, thus revealing their heterokaryotic nature.

Although these examples serve to show how heterokaryosis can be important in changes of pathogenicity, it must be emphasized that a heterokaryon is merely a temporary association of genetic material in separate nuclei, and in no way can it function as a system of permanent recombination between different genetic characters. This means that a heterokaryon cannot be expected to produce any change in pathogenic race that would persist after a sporulation stage in which uninucleate conidia are produced. Nevertheless, a system that depends on heterokaryosis as a first stage in its action has been recently found to give rise to more permanent changes in virulence.

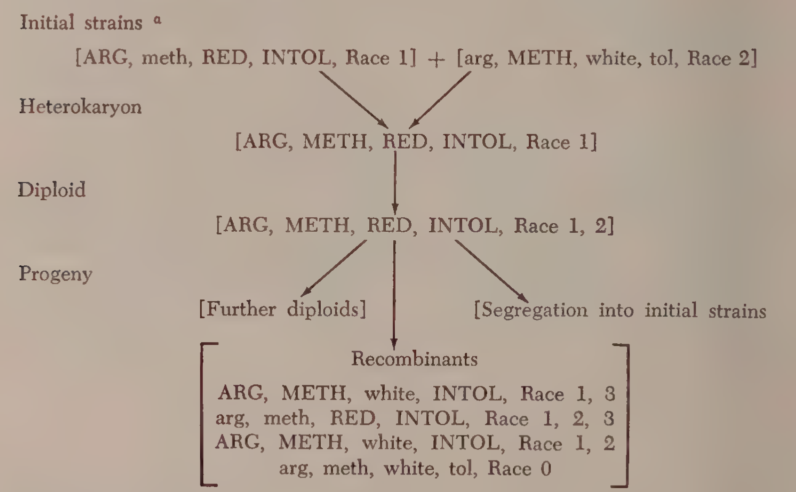
D. *Parasexual Recombination*

A system of genetic recombination in heterokaryons of imperfect fungi that operates without any sexual stage has been elucidated by Pontecorvo *et al.* (1953). During this process, a few nuclei arise which contain the combined characteristics of the different nuclei present in a heterokaryon. When these presumably diploid nuclei are isolated in single spores and analyzed, they yield not only duplicates of themselves together with segregants which are indistinguishable from the haploid components of the original heterokaryon, but also some completely new haploid types that recombine some of the properties of the originals. This process, now well established in aspergilli and penicillia (Pontecorvo *et al.*, 1953; Pontecorvo and Sermonti, 1954), has been called "the parasexual cycle" (Pontecorvo, 1956).

Buxton (1956) showed that new biotypes and pathogenic races can arise as a result of a similar parasexual mechanism in *Fusarium oxysporum* f. *pisi* (Table II). Heterokaryons formed between genetically marked isolates of pathogenic races 1 and 2 yielded single nuclei combining capabilities previously specific to each race, capabilities indentifiable by reference to the many genetic markers and by ability to infect differential hosts. These nuclei produced many recombinants, some of which had race 3 pathogenicity in that they were able to parasitize the pea varieties Delwiche Commando and New Era, resistant to race 1 and 2, and others were recombinants that were either nonpathogenic, race 1, race 0,3, or race 0,2. Similarly, recent work with rusts, although as yet incomplete, points to the tentative supposition that parasexualism could be an important factor in some of the changes in pathogenic race which occur in the absence of the usual sexual stage. For example, Watson (1957) has shown that 4 new races of *P. graminis* var. *tritici* arose from heterokaryons containing the two parentals, red race 111 and orange race NR-2. Similarly, Vakili and Caldwell (1957) found that

TABLE II

THE PARASEXUAL CYCLE IN *Fusarium oxysporum* f. *pisi*, SHOWING RECOMBINATION FOR SEVERAL LOCI, INCLUDING PATHOGENICITY DIFFERENCES. OTHER ALLELES ARE OMITTED FOR THE PURPOSE OF SIMPLIFICATION


Initial strains [a]
[ARG, meth, RED, INTOL, Race 1] + [arg, METH, white, tol, Race 2]
Heterokaryon
[ARG, METH, RED, INTOL, Race 1]
Diploid
[ARG, METH, RED, INTOL, Race 1, 2]
Progeny
[Further diploids]
[Segregation into initial strains
Recombinants
ARG, METH, white, INTOL, Race 1, 3
arg, meth, RED, INTOL, Race 1, 2, 3
ARG, METH, white, INTOL, Race 1, 2
arg, meth, white, tol, Race 0

[a] arg = arginineless
meth = methionineless
} Artificially induced markers

race type
RED
white
INTOL = intolerant to actinomycete
tol = tolerant to actinomycete
} Wild type markers

more new types arose from heterokaryons formed between the four genetically different nuclei in two uredospores than are theoretically possible. Thus, they recovered 16 known races and 17 completely new races of *Puccinia recondita* f. sp. *tritici* from a heterokaryon formed between the red race 2 and the yellow race 122. It seems that the most feasible explanation for the origin of the 17 new races is that they resulted from some kind of recombination between the characteristics of the four different kinds of nuclei in the heterokaryon. Both in rusts and in *Fusarium*, the new race relationships could arise by interchanges of chromosomes, by chromosomal inversions, or by similar mechanisms involved during the mitotic crossing over in the diploid nuclei within the heterokaryon.

E. *The Implications of Heterokaryosis in Fungal Taxonomy*

Because plant pathologists often need to retain live cultures of fungi for long periods under laboratory conditions, they have met with so many variations in their isolates that they have had good reason to doubt the usefulness of the currently generally used taxonomic criteria. So many changes in properties used in classification have occurred that pathologists have often become impatient with the reluctance of systematists to take into account the variability in living fungi. It should be recognized that variations caused by mechanisms of variability such as heterokaryosis are important criteria in any reorganization of the classification of fungi belonging to the traditionally "difficult" genera.

1. *The Dual Phenomenon*

One source of confusion in taxonomic descriptions could come from using heterokaryotic multinucleate conidia as first isolates, because this could result in segregation to cultures differing from the type species at the varietal or specific level. Different ratios between the numbers of the different nuclei of a heterokaryon can result in different semi-permanent cultural morphologies which in turn lead to a whole range of possible cultures, all deriving from one origin. Hansen and Smith's comprehensive study (1932) of heterokaryosis in *Botrytis cinerea* is only one of many examples which make nonsense of the use of certain cultural characteristics as taxonomic criteria. For instance, forty-seven cultures of *B. cinerea*, collected in California, yielded several morphologically distinct cultural types when subcultured as conidia. The spores and hyphae were shown to be ready-made heterokaryons that occurred as such in nature. The morphologically different strains frequently anastomosed and the resulting heterokaryons could always be broken down again into their original components. One important lesson to learn from this is that multinucleate spores cannot be regarded as individuals for taxonomic purposes but must always be treated as colonies that may have genetically different nuclei. Hansen (1938) further emphasized this point and produced additional evidence from an analysis of 916 wild-type isolates belonging to 30 different fungus genera, of which more than half had dual morphology when cultured from single spores on agar media. The most striking differences were between the conidial type (C) and the mycelial type (M); mixing M spores with C spores reproduced the MC types, which had an intermediate morphological appearance, and MC reverted to M and C when its single spores were cultured. This mixed condition, which Hansen called the "dual phenomenon," was a characteristic of the wild type in nearly all the imperfect fungi he examined. Similar results have since been obtained by many other observers, and the concept that many fungi must exist in nature with a ready-made pool of genetically variable characteristics is rapidly gaining ground.

If, as this evidence strongly suggests, isolates of fungi from nature are often heterokaryotic, or are aggregates of different clones of any given species, then the various culture media on which they are grown will act as a selective "bait" for their different components. It might seem hardly necessary to emphasize the importance of making taxonomic comparisons only between isolates that have been grown on identical media and under identical cultural environments, but unfortunately it is, for this elementary precaution has often not been taken. Consequently, it is not surprising that near chaos exists in the classification of many fungi that are important plant pathogens, and four of Hansen's examples serve to illustrate the extent of this inherent variability; *Verticillium albo-atrum* yielded 37 morphologically different strains out of 180 isolates from nature, *Botrytis cinerea* yielded 123 out of 300 isolates, *Ascochyta pisi* 3 out of 10 and *Phoma terrestris* 28 out of 100. Situations of almost equal complexity have been reported with others, for example, *Diaporthe*, in which two morphologically distinct strains, one diffuse and slow growing, the other compact and fast growing, readily anastomosed and frequently changed from one type to the other (Das Gupta, 1934). Mutation from one strain to the other initiated heterokaryons, which frequently segregated back to pure cultures of both components. Similar difficulties also occur in the Actinomycetes; isolates of *Streptomyces scabies* frequently anastomose with each other (Gregory, 1956) and heterokaryosis has been demonstrated in this organism by Bradley and Lederberg (1956). Ninety-one heterokaryons were obtained from 141 cultures of hyphal tips of colonies which arose from mixtures of genetically different spores. A genetic recombination system similar to the parasexual cycle has also been recognized in species of *Streptomyces* (Szybalski and Braendle, 1956).

2. *Heterokaryosis and Anastomosis between Different Species of Fungi*

Many attempts have been made to make heterokaryons between different species of fungi, but most have ended in failure (cf. Gossop *et al.*, 1940). This repeated failure could clearly be important in taxonomy, for incompatibility in heterokaryosis might be an aid to a better definition of the limits of doubtful species. An approach to this problem has been made by Cabral (1951) who, in attempting to assess the value of mycelial anastomosis as a taxonomic criterion, found that the established species of the existing system of classification were in fact always incompatible. In contrast to this, however, Taschdjian and Muskatblit (1955), in a study of the species of *Trichophyton* (the cause of Black Dot Ringworm in man) showed that *T. sulfureum* anastomosed with strains of *T. crateriforme* and with *T. sabourandii.* This led them to amalgamate all three species under the single species *T. tonsurans.* Although such conclusions need supporting evidence before they can be accepted, these preliminary steps may in time prove valuable as taxonomic aids, especially in fungi without a sexual stage and with which it is consequently impossible to demonstrate compatible interbreeding groups. In the absence of other reliable criteria, interanastomosing, or interheterokaryotizing groups of isolates might be usefully split off from each other as taxonomic entities. More studies of the kind done by Garnjobst (1953, 1955) on genetic incompatibility in heterokaryosis and anastomosis would prepare the ground for what could be a new approach to taxonomy, at least in the Fungi Imperfecti.

F. *Heterokaryosis as a Possible Means of Survival in Soil-Borne Parasitic Fungi*

By forming heterokaryons, fungi can adjust their genetic capabilities for colonization of many different kinds of substrate. This aspect of heterokaryosis might usefully be considered in any study of the many varied microenvironments that fungi encounter in a complex habitat such as the soil. It is not difficult to see how heterokaryosis could be a distinct advantage to soil-borne pathogens. For example, a pathogenic form of a fungus could exist as a component of a heterokaryon which was otherwise made up of nuclei of a saprophytic form of the same species (Buxton, 1954; Hrushovetz, 1957). This would enable it to survive for indefinite periods in soil as a saprophyte. Presented with a host, it could become pathogenic, and during this phase the nuclei containing genes governing pathogenicity would be favorably selected by substrates in host tissue or in the rhizosphere, and they would soon predominate in the heterokaryon. During invasion and the subsequent growth through the host tissue, nuclei of the saprophytic component might also enter within the one hypha. Indeed, there are indications that they do this, for saprophytic forms of soil-borne pathogenic fungi, in particular of fusaria, can often be isolated along with pathogenic forms in single hyphae growing from diseased host roots. Apart from this suggested mechanism, it is not easy to explain the presence of these saprophytic forms of fungi in the roots of diseased plants, especially when there is no evidence that they have made a separate later entry into already dead tissue.

The fluctuating existence from a pathogenic to a saprophytic mode of life is repeated in many soil-borne fungi each growing season, and there is no reason to suppose that the complex requirements for these two quite different phases of existence are governed by genes that are in the same haploid nucleus of a fungus. It should not be too difficult to devise experiments that would show whether readjustments similar to those already demonstrated *in vitro* could occur between nuclei governing either pathogenic or saprophytic activity in response to a host environment on the one hand, and to a soil environment on the other.

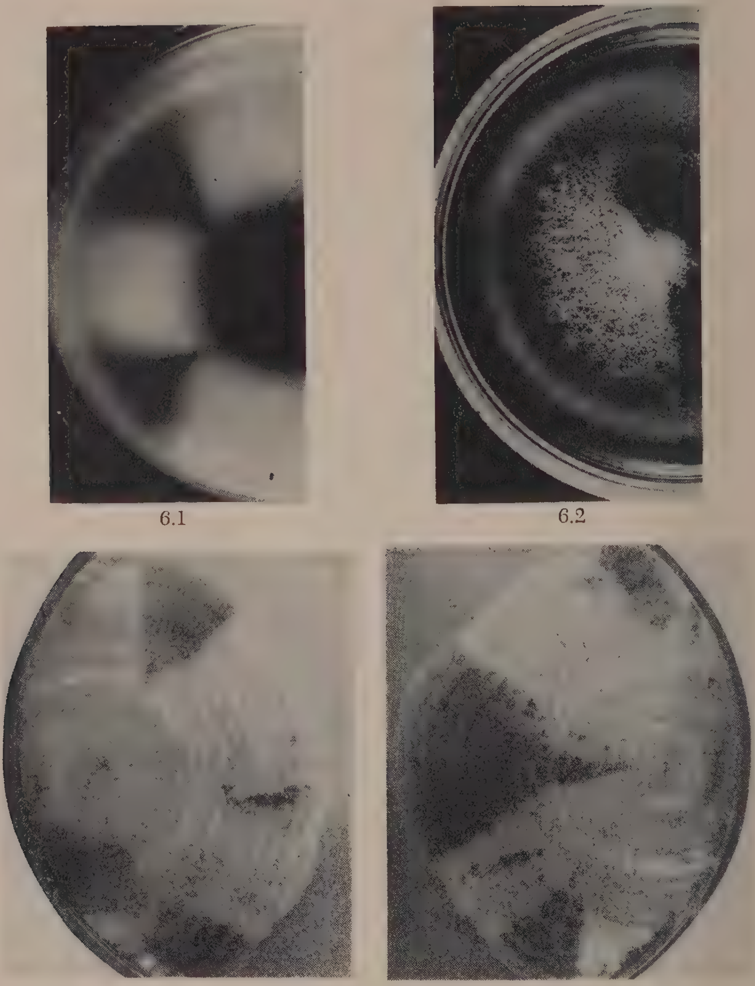
Selection of the different biotypes from wild type fungi can, of course, result from other causes than segregation of different kinds of nuclei. There is, in fact, no *a priori* reason for supposing that all the qualities in pathogenic fungi are governed by the nucleus. Recent evidence from work on *Aspergillus nidulans* indicates that the type of spore produced can be governed by self-reproducing inclusions of the cytoplasm, and these can be selected by manipulating the cultural environment (Jinks, 1954). Anastomoses between fungi obviously result in a mixing of their cytoplasms as well as of their nuclei, and because of this we could theoretically formulate an entirely new viewpoint on the nature of variation in fungi. However, with the recent quickened interest in these problems, we can claim to have made some progress since Brierley (1929) emphasized that "it is of primary importance that we should know the scope, direction, frequency and conditions of variation in fungi and bacteria."

IV. Saltation as a Cause of Variability

Having dealt with variations in fungi due to heterokaryosis, this is a convenient point at which to discuss what is perhaps the most controversial and least explained phase in the development of the study of fungi in culture. Saltation, often known as dissociation, or sectoring, is expressed by the appearance of morphologically distinct sectors in fungus colonies. When it occurs in bacteria, the dissociants arise as entire colonies that differ from the original isolates. Sectors in fungus colonies usually appear as fan-shaped growths if their growth rate is the same as that of the parent colony. When their growth rate is less, or the sector stops growing, they appear as "islands." Some sectors grow faster than the parent colony, when the edges of the sector curve outward and the parent colony may be overrun (Fig. 6).

Although saltation has been thoroughly described in many fungi, and especially in *Fusarium* and *Helminthosporium*, no author has so far produced critical evidence in favor of any particular genetic mechanism that might explain it. The investigations made by Stevens (1922), Christensen (1925), and by Christensen and Graham (1934) revealed that saltation was almost the rule in their cultures of *Helminthosporium*, despite the fact that the original isolates originated from single spores. However, because the spores of this fungus are multinucleate, there always remains the possibility that sectors are merely the expression of segregation of different strains from a wild type heterokaryon. Nevertheless, many uninucleate, or multinucleate yet homokaryotic, conidia from different fungi often give rise to saltant cultures. From this it might be assumed that saltation could be caused by mutation were it not for a good deal of evidence to the contrary.

First, are saltations persistent when subcultured? Stevens (1922), from his work on sectors of *Helminthosporium* that differed from the original isolates in color, spore size and shape, growth rate and density of growth, found that while some remained stable after subculture, others tended to saltate again. Mohendra (1928) found that sectors of *Phoma, Neocosmospora,* and *Alternaria* did not revert to the parental type, whether subcultures were made from young or old mycelium, or from spores. Second, are sectors affected by culturing on different media, and does the type of medium used affect the rate of saltation? Tyler (1938) found that *Sphacelotheca* sectored more on malt agar media containing nitrogenous salts than it did on potato dextrose agar, sugar media with no salts, or on peptone-containing media. In addition, among fourteen sectors which had remained constant on potato dextrose medium for more than a year, eight sectored when they were transferred to malt agar medium. If mutation were responsible for these changes, it could hardly have occurred at random, but would seem to be induced by changes in the cultural environment. Mitra (1931) found that more sectors occurred when cultures of *Helminthosporium* were grown on shallow media than on deep media, which again argues against the assumption that mutation is solely responsible for sectoring. Similarly, Brown (1926, 1928) showed that the rate of saltation in *Fusarium* was



6.1 6.2

6.3 6.4

Fig. 6. (1) *Fusarium oxysporum* sectoring to give differently colored variants. (2) *F. oxysporum* producing a sector with increased growth of aerial mycelium. (3 and 4) *Helminthosporium sativum,* showing sectors for color, differences in growth rate and heavier sporulation capacity (Stevens, 1922).

influenced by the type of culture used. If, as it seems from most of the evidence, culture variants arising by saltation from single spore cultures are more often than not irreversible and hereditable, then mutation, in a general sense, would explain their origin. When, however, changes in the culture medium can greatly affect the rate of saltation, then the mutational theory is no longer tenable. A more likely explanation is that heterokaryons, probably originating as a result of a mutation early in the life of the saltating culture, go on to express a number of different morphological appearances as a result of changes in their nuclear ratios, these having been brought about by changes in the nutrient status of the culture medium. There are, of course, other explanations that could equally well be founded on the possibilities of morpholgical differences being controlled by hereditary particles in the cytoplasm, or even by assuming the presence of a parasexual cycle. Stevens may indeed have been near the truth when he suggestd that saltation was probably caused by a mechanism resembling the "bud variation" described by De Vries in higher plants, where it is now known that interchanges between chromosomes in ring formation result in a higher rate of production of progeny that differ from the parents than would mutations at the gene level.

A. *The Implications of Saltation in Fungal Taxonomy*

What measure of reliability can be placed on the taxonomic characteristics of fungi that seem so unstable in culture as do *Fusarium* and *Helminthosporium?* One notable attempt to answer this basic problem was made by Brown (1926, 1928), as a result of his extensive study of saltation in *Fusarium*. Among 40 strains which he obtained as sectors, he found that many differed from the parental cultures in the size and septation of their spores, and this constituted a serious objection to continuing to use such features as important criteria in classifying the fusaria. Indeed, many saltants from one *Fusarium* species looked so much like those from another that it became quite clear that the taxonomy of the whole genus needed urgent revision, and Brown proposed that many of the existing species should be merged. Similarly, Presley (1941) found that all grades of morphological types occurred among sectors in isolates of *Verticillium* from *Chrysanthemum*. He, therefore, suggested that the genus *Verticillium* could not be divided into the species normally considered to be valid. Purdy (1955) also concluded that the variations, again expressed as sectors, in *Sclerotinia sclerotiorum*, *S. trifoliorum*, and *S. minor* meant that they were taxonomically inseparable. In these and many other fungus genera, it would be a considerable help if a taxonomic system were proposed that took the inherent variability into account, rather than continue the outdated system of classifying with reference to the features of dead type specimens that can no longer express their variability.

B. *Saltation and Pathogenicity*

The important effects of saltation on pathogenicity have been most extensively studied in *Fusarium*, and the results have been no less conflicting than with changes in morphology. Haymaker (1928) had two strains of the tomato wilt fungus, *Fusarium oxysporum* f. *lycopersici*, one constant in culture, the other always tending to saltate and differing from the other both in appearance and in pathogenicity. The differences in pathogenicity caused by saltation proved to be in virulence only, for no new pathogenic races could be demonstrated among the saltants. Like many other workers, Haymaker tentatively considered that the saltants were induced by changes in the cultural environment. Snyder (1933) described 15 different strains of the pea wilt *Fusarium*, again differing in virulence but not in race, and he supposed that the differences were probably the result of some type of sexual mechanism which might occur in nature, or that they were the result of one which had occurred at some earlier stage in the evolution of the fungus. In a study of pathogenic changes in cultures which arose as sectors in the tomato wilt *Fusarium*, Wellman (1943) found that, among several which differed from the parent culture in pathogenicity, rather less than 1 in 1000 had increased in virulence. Nevertheless, a rate as high as this means that saltation can be an important factor in the evolution of parasitism. Wellman gave a third alternative explanation of the basis of saltation, for he inclined to the view that the changes in his isolates were more concerned with adaptation than with mutation. Weindling's (1939) experience with *F. vasinfectum* from cotton also indicated that nearly all the variants from the parental isolates tended toward reduced virulence, but he had no comment to make on the nature of the mechanism responsible. Burkholder (1925) found that *Fusarium martii phaseoli* (a cause of bean wilt) dissociated during the 5 years it was kept in culture. His original isolates, which were "blue-green and slimy," changed to "white and fluffy," and there were also considerable changes in the relative proportion of the two different spore types and in the extent of their septation. The 5-year-old cultures could still infect beans but their virulence had decreased by half. However, passage through beans restored the original cultural appearance but not the original pathogenicity. LeClerg (1939), by contrast, found that sectors of *Rhizoctonia solani* from sugar beet produced more damping-off in seedling beets at 15° and 25° than did the parent cultures, which, however, more readily rotted beet roots. Changes in culture among pathogenic fusaria were found by Oswald (1949) to be accompanied by a decrease in virulence. His sporodochial type from four fusaria changed to a pionnotal adpressed form in culture, a state from which they never reverted (Fig. 7).

As in Brown's work some variants were morphologically intermediate between the species limits of the four isolates used, again emphasizing the far-reaching effects saltation may have, not only on pathogenicity,

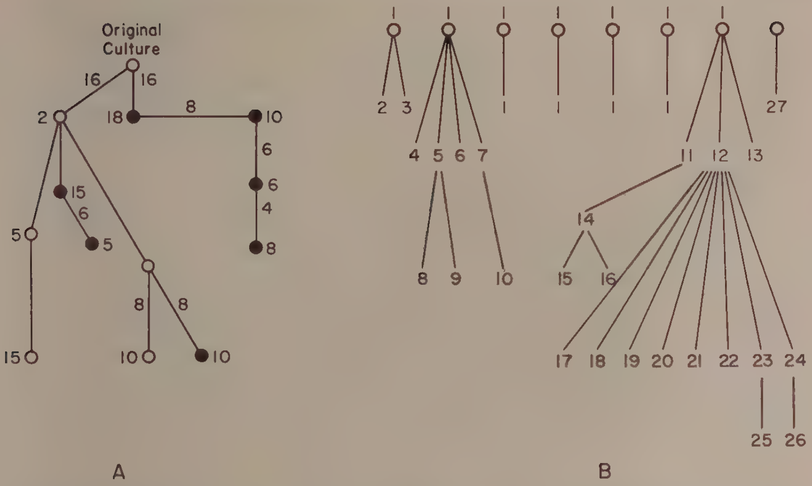


FIG. 7. Pedigrees of sectoring and dissociation in cultures of *Fusarium* (A) and *Helminthosporium* (B): (A) Two cultural types, ○ sporodochial, ● pionnotal, arising in the subcultural progeny of a single-spore culture. Numbers by the lines show the interval between subcultures in weeks, others refer to the number of subcultures made. (Adapted from Oswald, 1949.) (B) The pedigree of morphologically different sectors arising from eight single-spore cultures of common origin. Each number represents a morphologically different isolate. (Adapted from Stevens, 1922.)

but equally on taxonomy. Cormack's results (1951) from a study of two fusaria from alfalfa agreed with Oswald's, and his saltants, in addition to being less virulent than the parent cultures, were also less able to survive in dried soil.

C. *Attenuation*

As already stated, virulence of pathogenic fungi commonly decreases when they are kept in culture or when passed through various hosts, and such changes, whatever their genetic background, can ultimately lead to complete loss of virulence. Whether the losses have been permanent or temporary, they have been collectively described as "attenuation." This term has more often been used to describe the changes in virulence in viruses during host passages than it has in fungi, but in the latter it also includes those changes in pathogenicity that have been called dissociation.

A clear distinction must be drawn between the probable mechanisms governing attenuation in fungi and in viruses. In fungi, loss of pathogenicity can occur in cultures which originate from one uninucleate haploid single spore, the use of which greatly increases the probability of starting with homogeneous inoculum. With viruses, however, if only for the reason that a great many virus particles need to be in the inoculum before a single lesion can be obtained, the risk of starting with mixed inoculum is obviously greater. Moreover, a supposedly pure inoculum of virus seems more easily able to become heterogeneous as a result of what appears to be mutation.

With viruses, and to a lesser extent with fungi, the very high rate of reproduction when inside a suitable host rapidly leads to millions of progeny, either as virus particles or as fungus nuclei. Even a relatively low mutation rate might, therefore, be expected to result in the appearance of one or more variants within a single host plant and such variants may differ in virulence from the original inoculum. Although the analogy may be false, judging by the mutation rates in the fungus *Neurospora crassa,* or in the fruit fly *Drosophila melanogaster,* it is at least possible that the changes in virulence in viruses can be caused by mutation. In fungi, however, too little is known of the rate of increase of nuclei, once the fungus is in the host, for any conclusions to be drawn. Moreover, other mechanisms of variation that have been shown to exist in fungi can produce similar variants, and often at a rate more in keeping with the frequency with which they seem to arise in nature.

There are many examples of attenuation in viruses, and heat treatment and host passage have been repeatedly shown to be effective. For example, Johnson (1947) found that after tobacco mosaic virus had been passed through sea holly *(Eryngium aquaticum)* it produced only mild symptoms on tobacco. The sea holly had presumably acted as a selective filter separating the mild and severe forms of the virus that Johnson considered had arisen by mutation from severe to mild. A similar explanation could also apply to many dissociative changes in fungi where mutants occurring early during growth would be differentially selected by different cultural or host environments; examples of this are given in the next section of this review.

Bacteria are commonly attenuated when kept in culture, as Kelman and Jensen (1951) found with *Pseudomonas solanacearum*, which causes plant wilts. Their cultures rapidly decreased in virulence during successive transfers, but did not do so when kept in sterile mineral oil. Not only changes in virulence, but also in tolerance toward changes in pH of the culture media and in morphological features, were found by Ark (1937) in *Erwinia amylovora*, the cause of the destructive fire blight of pear. Older cultures tended to dissociate into rough forms, and rhizoid or translucent variants were also encountered. Ark described dissociation as "the idea of regular cycles through which bacteria pass in their life history" and he pointed out that it was an important factor in the rise and decline of fire blight outbreaks.

In yeasts, the dissociations observed by Fabian and McCullough (1934) occurred in a well-defined sequence. By altering the temperature and light intensity, their cultures could be repeatedly made to follow a specific line of variation, and the resulting variants always remained stable, never reverting to their original forms. Their work almost implies that dissociation is the outcome of some regular recombination system rather than of randomly occurring mutations. It is, in fact, reasonable to suppose that many of these examples of saltation, dissociation, and attenuation will ultimately be assigned to some more specific genetic mechanism of microbial variation. Indeed, the only useful purpose that has been served by all three of these categories during the rise of the genetic approach to plant pathology has been that of giving a temporary name to general examples of unexplained variations. A good measure of future progress in the genetics of variation in plant pathogens will be the rate at which these terms are discarded and the relevant phenomena assigned to more specific genetic mechanisms.

V. Adaptation as a Mechanism of Change in Plant Pathogens

Adaptation in microorganisms has been known since 1887 when Kossiakoff noticed that bacteria could alter their tolerance to antiseptics. Not only is its practical importance now fully recognized in medicine, bacterial physiology, and industrial microbiology, but it is also accepted as an important mechanism of microbial evolution.

In the broadest sense, adaptation in microorganisms is the acquisition by an organism of the ability to perform some physiological process which it could not previously fulfil, or at least could not do effectively. Adaptations in microorganisms have been shown by three main lines of work: (1) acquiring a tolerance toward previously toxic substances, (2) acquiring the ability to use new substrates for growth, (3) changing in virulence toward host plants.

The first of these is obviously important in medicine and plant pathology because it leads to the development of forms resistant to antibiotics, drugs, and fungicides. The second has been demonstrated both in academic studies of microbial physiology and in industrial fermentation, in which there are many examples of microorganisms undergoing adaptive changes in their basic nutritional needs. It is this type of adaptation that is most closely allied in principle to the ability of some plant pathogens to alter their pathogenicity, for the ability to use new sources of food supply could clearly be a prerequisite for a change in virulence. However, it is equally probable that some disease resistance in plants may be controlled by specific host-produced toxicants. Many experiments have been made to test for adaptive changes in fungal and bacterial pathogenicity, and it is not surprising that conflicting results, or results capable of many different interpretations, have been obtained.

A. *The Basic Mechanisms of Adaptation*

Before dealing with adaptive changes in plant pathogens, it is necessary to discuss the inherent difficulties in discriminating between the possible interpretations of the mechanism underlying microbial adaptation. Take a simple example of an organism which cannot grow in a substrate S, but the possession of an enzyme Es would enable it to do so. It has been repeatedly found that, by keeping the organism in S for a period of time, or by successively subculturing it into increasing concentrations of S, the organism will gradually acquire the ability to grow in S, and furthermore, the presence of Es can often be demonstrated. There are two principal explanations for this adaptive behavior. One invokes the gradual formation of an enzyme that can break down S and so make it available for growth of the organism; this process is called "enzymatic adaptation," as distinct from the more general term "adaptation." The other discounts this view and postulates, often with good evidence, that mutants arise that have the ability to use the metabolites in S, that such mutation probably occurs at random, and that the mutants are then favorably selected by S. This would lead to the gradual building-up of a population which could use S, and the end result would be the predominance of a new type of organism, no less than as a result of enzymatic adaptation. Here adaptations that are caused either by the selection of mutants or by the appearance of adaptive enzymes will be considered. In many experiments, the adaptations have proved to be only temporary, whereas in others they are permanent and hereditable.

Because of these alternative explanations, many experiments have been designed to detect mutants that may arise while the test organisms are in the substrate. The alternative mechanism based on an adaptive enzyme, which involves some as yet unknown change in the internal economy of the cell, either nuclear or cytoplasmic, and which results in the gradual production of an enzyme, can only be inferred either when mutation can be shown not to occur or when it can be taken into account by a critical technique. Ryan (1952) claimed that mutation, at a rate of 2×10^{-7} per bacterium per generation, enabled a strain of *Escherichia coli* previously unable to use lactose (lac$^-$) subsequently to do so. Mutations towards lac$^+$ ability arose quite independently of the substrate in which the bacteria were kept, and the distribution of the lac$^+$ individuals in the different cultures used in the experiments was the same as that which would theoretically be expected if they had in fact arisen by random mutation. Ryan dismissed the alternative explanation that the acquired and inheritable lactose-fermenting ability was brought about by a system of enzymatic adaptation, and he argued that much of the earlier work on adaptation in *Escherichia* should be reinterpreted as mutation followed by selection.

The role of the substrate, then, is either to select mutants as they arise or to induce the formation of enzymes. If enzymes are already present but in too small a quantity, or are inhibited in some way, the substrate may induce them to work. In addition, there is the possibility that some substrates may be mutagenic, any suitable mutants again being selected as they arise. There is no reason why enzymatic adaptation itself could not consist of a series of mutations, each with a very small effect, but successively acting in one major direction, ultimately leading to the full development of a specific enzyme. However, the cause of many adaptations may be a combination of both processes, involving the chance occurrence of mutant progeny, with new physiological properties, which can then produce a particular adaptive enzyme.

Hinshelwood (1953) and Dean and Hinshelwood (1953) criticized the mutation theory of microbial adaptation in the light of their studies on the acquired tolerance by bacteria to drugs. Their arguments were based on the fact that mutation alone could not account for the rapidity with which most adaptive changes occur. In addition, in experiments in which progressively increasing concentrations of substrates are used to induce adaptation, an orderly, and unlikely, succession of mutants would have to be postulated to account for the different degrees of adaptation attained at each stage of subculturing. Stanier (1951, 1953) also emphasized the first of these considerations and pointed out that the occurrence of a few mutants at an early stage in a relatively large population of individuals in an inducing substrate would lead to a growth characterized by a long lag phase, before the mutants could develop in sufficient numbers to affect the population. Hinshelwood (1953), however, drew attention to the difficulties that can arise when interpreting adapting cultures that have different lags, as shown in Fig. 8.

The ratio of n_2 to n_1 is much greater than would be expected from the relatively little difference between the means A and B of the two distribution curves of lags, the one curve showing a normal distribution, the other a preponderance of short-lagged cultures. Taking the means of lags only would, therefore, tend to obscure the fact that there was a greater proportion of short-lagged colonies in one set *(A)* than in the other *(B)*.

Although the mutation and enzymatic adaptation theories are opposed, they are not diametrically so, for both basically depend on the occurrence of mutations, though of different degree. The point really at issue is the extent and nature of the mutations and the direction of selection or adaptation they may undergo once they have arisen.

Adaptation, whatever the mechanism underlying it, allows the environment, often a specific substrate, to exert a far reaching effect on microorganisms. Obviously, the effect must be exerted through the cytoplasm and, when changes are permanent and inheritable, the nucleus itself might be altered. By analyzing the progeny of adapted cultures it is often possible to distinguish between cytoplasmically inherited characters on the one hand, and nuclear genes on the other. Interesting examples of acquired changes that are similar to adaptations have been demonstrated in bacteria that can acquire some of the properties of different, but related, strains when grown in their culture filtrates or cell extracts. This process, first noticed by Griffith (1928), and known as transformation, has been recently described in pneumococci by Ephrussi-Taylor (1951), who found that highly purified solutions of pneumococcal deoxyribonucleates influenced the changes of colony morphology between rough and smooth types, a fact that may imply that the changes were genetically controlled.

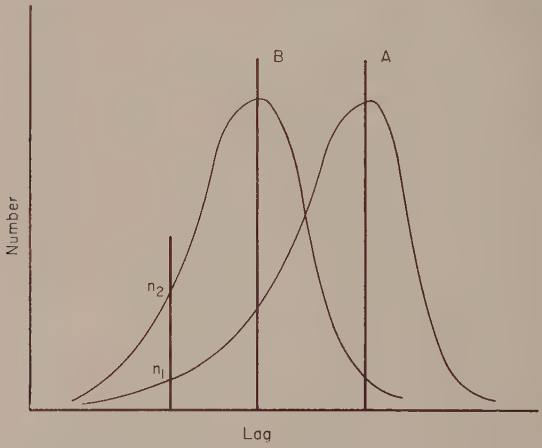


FIG. 8. Distribution of lags in adapting colonies of microorganisms (Hinshelwood, 1953).

Adaptive changes have been considered as a possible method of variation in plant pathogens and, indeed, there are many proven examples both in pathogenic fungi and bacteria. Because the different interpretations of adaptive changes in fungi need frequent revision in the light of advances in genetic knowledge, it is best to consider the earlier examples of adaptive parasitism separately from the more recent work.

B. *Early Examples of Adaptive Changes in Fungi*

Some of the first observations that environment could modify the pathogenicity of viruses, bacteria, and fungi were made in researches in human and veterinary pathology. For example, Pasteur found that rabies virus considerably increased in virulence after twenty or more passages through rabbit brain. Conversely, reductions in virulence can be obtained by passing pathogenic organisms through noncongenial hosts, and this important discovery is used for producing attenuated viruses for immunization. This "host-passage effect" was extensively tested in fungi by a group of plant pathologists led by Marshall Ward at the end of the last century. Ward (1903) found that the specificity of pathogenic races of *Puccinia dispersa* could be changed by keeping them in association with certain grass hosts. For example, although a race could not be transferred directly from one species of *Bromus* to another, it could be transferred by first passing it through a third species (Table III).

TABLE III
HOST PASSAGE EFFECT ON INFECTION BY RUSTS ON SPECIES OF *Bromus*[a]

Host	Spores from *B. sterilis*		Spores from *B. mollis*	
	No. of plants infected	Successful infections	No. of plants infected	Successful infections
B. gussonii	60	37	53	6
B. krausii	29	14	27	27
B. molliformis	25	1	26	2
B. pendulinus	53	12	50	30
B. vestitus	4	1	4	3

[a] Ward (1903).

In *Bromus Gussonii* there were only 6 infected plants out of 53 inoculated with spores from rust on *B. mollis*, but 37 out of 60 were infected when spores came from the same rust after being inoculated to *B. sterilis*. Ward cited many more examples of the effect of host passage, and he called the intermediate species "bridging hosts." His concept of these changes in rusts came to be known as "the bridging host theory."

Salmon (1904) obtained results similar to Ward's with his experiments on mildews of grasses. Among many isolates of *Erysiphe graminis* he differentiated a number of pathogenic races by their reactions on different species of *Bromus*. The *B. racemosus* race could not at first infect *B.*

commutatus, but after infecting *B. hordeaceus*, which was the common host for races of rust able to infect the other two Bromes, it became able to do so (Fig. 9).

In a similar study of what appeared to be host-induced changes in pathogenicity, Massee (1904) found that the saprophytic fungus *Trichothecium candidum* could be gradually induced to become pathogenic on leaves of *Begonia*. When spores were used as inoculum on a leaf which had been injected with a solution of 2% cane sugar, very small but nevertheless sporulating lesions appeared. The few spores produced were transferred to another injected leaf and so on. After twelve successive transfers from small lesions the fungus became increasingly virulent to *Begonia* until, after some fifteen transfers, it caused extensive lesions without the need for injections of cane sugar. This transition from a saprophytic to a pathogenic existence took 12 weeks, while isolates succcessively transferred to uninjected leaves, as controls, caused no lesions.

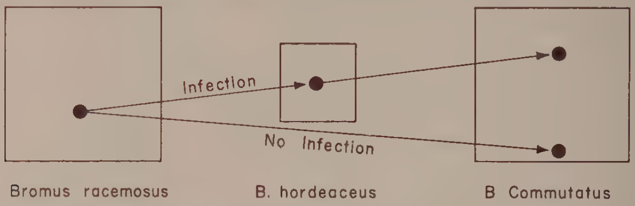


FIG. 9. Diagram illustrating the "bridging host theory" of adaptive parasitism. (Salmon, 1904.)

Many explanations have been advanced to explain these changes in fungi and many attempts have been made to reproduce them. Freeman and Johnson (1911) successfully repeated Ward's work, when they found that barley could serve as a bridging host for *P. graminis* var. *tritici*, which was thereby induced to grow on oats. In a short time, several other investigators had also produced good evidence in support of the bridging host theory, but there were notable exceptions. When Stakman *et al.* (1918) tried to get bridging host evidence with races of *P. graminis* var. *compacti* from *Triticum vulgare*, they consistently failed to do so, for neither barley nor club wheat would act as a bridging host, although they appeared theoretically suitable for the purpose. In addition, barley and resistant wheats were inoculated several times in succession, but the rust never increased its virulence toward them.

This demonstration of the apparent constancy of biological forms of rusts served to conclude the early phase of the work on adaptation in pathogenic fungi, and many workers now explain Ward's results either by assuming that his original inoculum must have contained a mixture of races, or that it mutated at a high rate to other degrees of pathogenicity. Nevertheless, the first supposition might be dismissed, for Ward took great care to purify his inoculum and to exclude contaminants, and the second has not been borne out by recent experiences of changes in the pathogenicity of rusts. In any case, an unusually high mutation rate would be needed to explain the rapidity of the changes observed in the bridging host experiments. More recently, Bean *et al.* (1954) found that all their physiologic races of *P. dispersa* were remarkably stable in their infection types and host range. As a result of 14 years' work on Brome rusts, they found no support at all for the bridging host theory, and they stated that Ward could have been seriously misled by failing to recognize that new races of the rust were constantly arising.

C. *Recent Investigations of Adaptation in Plant Pathogens*

Stimulated no doubt by recent work on adaptation in the physiology of bacteria, mycologists and plant pathologists have recently begun to reexamine fungi for this character. Two major directions have been taken, one dealing with adaptation of fungi under cultural conditions, the other with adaptation in pathogenicity.

1. *Acquired Tolerance to Poisons in Culture*

A knowledge of adaptation of fungi under cultural conditions is especially important in considering inhibition by growth products of other organisms and by fungicides. One example of a fungus that can adapt toward tolerance to toxins is *Ustilago zeae* which, after ten successive transfers, increased its tolerance to inhibition by sodium arsenite in agar media from 2400 parts per million to 7000 p.p.m., (Stakman *et al.*, 1946). However, when the adapted colonies were returned to an arsenic-free medium they soon lost their acquired tolerance. Wilson (1947) did similar experiments with *Sclerotium rolfsii* and *S. delphinii* and found that only five transfers, over a period of 10 months, were necessary to allow them to acquire tolerance to arsenite in concentrations which were increased from 125 p.p.m. to 150 p.p.m. When the "trained" cultures were returned to media containing 125 p.p.m. of arsenite, their growth rate was twice that of untrained cultures. Christensen (1946) also found that monoconidial lines of *Gibberella zeae* developed increased tolerance to malachite green, mercuric chloride, and ethyl mercury phosphate. Unfortunately, few investigators have advanced any explanation of their results. but Christensen and Daly (1951) in a discussion of the problem, inclined toward the view that mutations were responsible for most of these apparently adaptive changes. Nevertheless this view only seems the more plausible one because the alternative theory based on enzymatic adaptation does not at first sight seem applicable to changes in tolerance towards poisons. However, some fungi may have a detoxification system which can adapt and so could alter them to grow better in the presence of poison. Moreover, a theory based entirely on mutation again does not fully explain the readiness with which the altered cultures revert, as most do, when put back on media containing subtoxic concentrations of inhibitory substances.

Fungicides containing copper have consistently proved successful in disease control, but Mader and Schneider (1948) reported acquired tolerance by monoascosporic cultures of *Sclerotinia fructicola* to copper sulfate in agar media. All five of their original isolates could just tolerate a concentration of 7000 p.p.m., but after 18 weeks' growth on 5000 p.p.m. they became able to tolerate 10000 p.p.m. While some reverted to parental type when later cultured on a copper-free medium, others remained tolerant to the higher concentration. In addition, these copper-tolerant cultures differed from the original copper-susceptible cultures in their capacity to cause fruit rot, but no details of the differences were given. In a similar study, Hirt (1949) found that, on an agar medium containing 0.1% copper, normally toxic to fungi, one in ten of his isolates of *Poria xantha* was able to grow, and these continued to do so after subculture. In this example, copper tolerance was linked with a morphological characteristic, the possession of compact hyphae. His results also indicate that there are naturally occurring clones of copper-tolerant strains of *Poria*, which were selected by an agar medium containing copper. However, the examples of increased tolerance to poisons by *Ustilago*, *Gibberella*, and *Sclerotinia* cannot be explained on a similar surmise, for it took several generations before tolerant strains appeared; if tolerance had been a wild type characteristic, the first subculturings should have revealed it.

It is well established that some bacteria pathogenic to man can adapt to become tolerant to antibiotics, and the same applies to some plant pathogenic bacteria. Carmono-Gomez (1956) found that the plant pathogenic bacteria *Xanthomonas phaseoli* var. *fuscans* and *X. vignicola* could be trained to grow in concentrations of an antibiotic as high as 300 μg. per milliliter if successively transferred 9 times, at 10-day intervals, in a series of increasing concentrations of the antibiotic (Fig. 10).

One example in which mutation could have been directly responsible for acquired tolerance to a poison was recorded by McKee (1951). *Fusarium coeruleum* sectored profusely when grown in agar in petri dishes which had 0.01 gm. tetrachloronitrobenzene (the active principle of a dressing applied to newly dug potatoes to prevent dry rot in storage) stuck inside the lid. Twenty-eight sectors arose in ten of thirteen dishes and many of the cultures obtained from these sectors could withstand the hitherto toxic effects of the vapor, and the newly acquired tolerance was unaltered by passage through potato. In this example, either the chemical acted as a powerful mutagenic agent or it exerted a strong selective effect on mutant clones already existing in the original isolates.

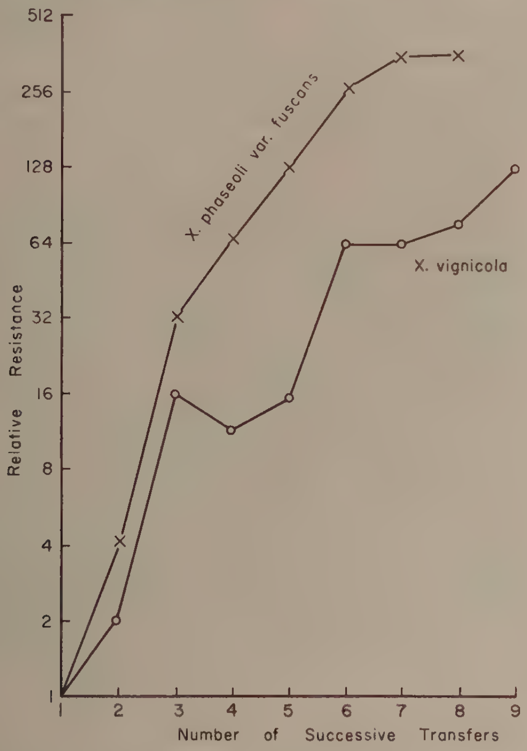


FIG. 10. Induced resistance of *Xanthomonas vignicola* and *X. phaseoli* var. *fuscans* to antibiotic A-6 after successive exposure to a series of increasing concentrations of the antibiotic. (After Carmona-Gomez, 1956.)

2. *Adaptations in Pathogenicity*

Adaptation in fungi not only plays a part in changes of virulence of biotypes, but it sometimes results in a change of pathogenic race. As already pointed out, it is at least probable that the earlier workers, Ward, Salmon, Freeman, and others, were dealing with examples of race change by adaptation.

One example of adaptation to a new host is that of the relationship between phage and bacteria. Luria (1953) described what he considered were nonmutational changes which were strictly phenotypic and nonhereditary, and were determined by the bacterial host cell in which the virus was produced. For example, phage P_2, which grew in *Shigella dysenteriae* strain Sh, could grow in every cell of it, but could only grow in 1 in 10^4 cells of *Escherichia coli* strain B. When the phage was grown in B, it adapted so that it became able to grow in all the cells of B. However, when grown in Sh again, it became restricted to 1 in 10^4 cells of B.

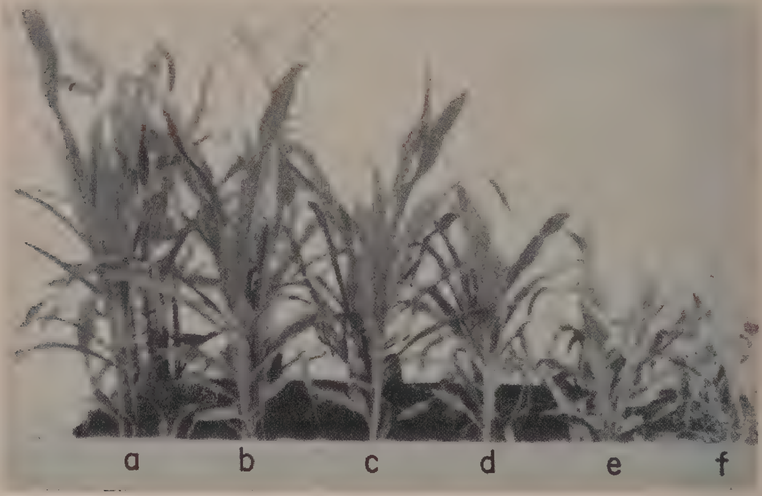


FIG. 11. Successive passages of S15 stock culture of *Phytomonas stewarti* through resistant maize host OSF. (a) check; (b) inoculated with stock S15; (c), (d), (e), (f) respectively, inoculated with S15 passage strains after two, four, six, and eight successive host passages. (Lincoln, 1940.)

Recent work has similarly demonstrated that certain pathogenic fungi and bacteria can change their virulence during passage through resistant or susceptible hosts. Studies with *Phytomonas stewarti*, the cause of bacterial wilt of maize (Fig. 11), demonstrate this phenomenon (Wellhausen, 1937; Lincoln, 1940). While the virulence was increased by successive passages through a highly resistant host, it was decreased by successive passages through a susceptible one. However, this example of apparent adaptation could have been caused by selection of mutants that may have arisen during multiplication of the bacterium in the host.

Lincoln (1940) extended this work and traced the changes in virulence which took place progressively over the period of time the bacterium was in the maize plants. In addition, he demonstrated the effect of selection, both by resistant and susceptible varieties of maize, in artificially mixed cultures of virulent and avirulent bacteria (Fig. 12).

In resistant plants, the proportion of avirulent strain probably decreases as a result of the presence of inhibitors, or the lack of some essential metabolite. In susceptible plants, the avirulent strain may increase by adaptation; in either case, adaptations would result in mixtures of virulent and avirulent strains, which are then selected by the different environments of resistance or susceptibility. Lincoln also showed that thirteen single-cell cultures of the bacterium altered their virulence during host passage; thus an adaptive change might occur, again because of the possibility of mutation, as a direct result of host passage, rather than by the selection of previously existing variation in the inoculum.

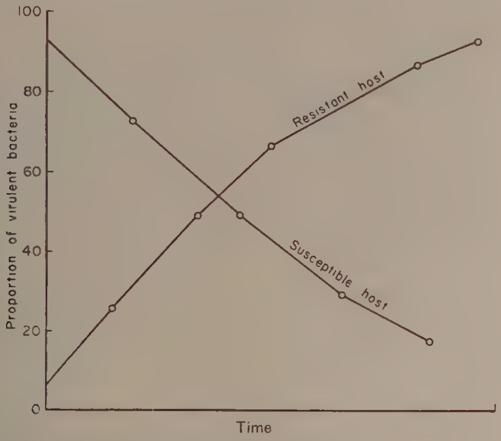


Fig. 12. Effect of host passage on proportion of virulent to avirulent bacteria in mixed culture inoculations. (After Lincoln, 1940.)

A test of Wellhausen's theories was made by Reddick and Mills (1938), who examined the relation of *Phytophthora infestans* to a series of potato varieties. These were arranged in order of increasing resistance to late blight. Starting with cultures of uninucleate zoospores, and hence avoiding the possibility of using heterokaryotic inoculum, they first grew the fungus on the highly susceptible variety Green Mountain. Successive transfers were then made to the varieties Evergreen, slightly resistant; President, considerably resistant; KB/5, usually immune but sometimes bearing lesions; ET/9, always immune and never bearing lesions. During the transfers, the isolates progressively increased in virulence and gradually became able to cause lesions on variety KB/5.

Even after they had been reinoculated to the susceptible variety Green Mountain, their subsequent newly acquired virulence to the resistant KB/5 remained unimpaired. Mills (1940) also found that the potato strain of *P. infestans* could be induced to attack tomato, which is normally resistant to it; after seven passages through tomato plants, its virulence toward tomato plants had increased so much that it killed them. More passages through tomato, from eight to twenty-two had no further effect. Both potato and tomato strains attacked potato with equal vigor, but tomato strains did not change in virulence toward tomato even after being kept for 3 months on potato leaves or for 6 months on tubers. Mills suggested that the tomato strain arises in nature as a result of the passage of the potato strain through tomato, and this theory is supported by the observation that under field conditions the tomato strain always appears a few weeks after the potato strain. He repeated these tests, and got the same results, on 6 different occasions. Both De Bruyn (1947) and Ferris (1955) have confirmed that association with a resistant potato variety can alter the virulence of the blight fungus. Ferris took two blight-resistant potato varieties, one duplex for resistance gene R_1 (R_1R_1), the other duplex for resistance gene R_2 (R_2R_2). When susceptible simplex plants (R_1 or R_2) were inoculated with either race 1, race 1,4, or race 2, severe lesions occurred, whereas the duplex plants bore only small lesions. However, a more severe susceptible reaction occurred on the duplex plants when the inoculum came from a culture which had been reisolated after a series of passages through plants of their own genotype (R_1R_1 or R_2R_2). This "passage effect" again shows that *Phytophthora* can alter its virulence toward a hitherto resistant host, the phenomenon seeming essentially similar to the "bridging host evidence" revealed earlier by Ward (1903) on *Bromus*. One striking feature of Ferris's work is that a second passage through duplex-resistant plants induced an even greater increase in virulence than did the first passage, and this makes it more reasonable to assume that the virulence was altered by a change in enzyme activity, adapted to a new level, rather than to postulate a system of random mutation which by some chance resulted in different levels of acquired virulence.

Despite these examples of the adaptive tendencies of some plant pathogens, other workers have reported failure to demonstrate the phenomenon. For example, Keitt and Langford (1941) were unable to alter the virulence of three haploid lines of *Venturia inaequalis* by passing them through apple on four successive occasions. In addition, Bonde *et al.* (1940) considered that there was no circumstantial evidence in the field for the adaptability of *Phytophthora infestans*, at least in their experiments. Nevertheless, there is at present more evidence for adaptive change in virulence than there is against it, and the difficulties really lie in assigning the correct interpretations to the mechanisms underlying the adaptations.

Although much has been gained from this increasing body of evidence for adaptive pathogenicity, a more specific relationship between host resistance and some chemical substance produced by the host needs to be established before much further progress can be made. One example of this is provided by Gäumann and Bohni (1947) who demonstrated adaptive enzyme production in *Aspergillus niger*. The fungus produced pectinase only after it was kept on a medium containing pectin, and no enzyme was formed in control treatments in which it was grown on a pectin-free medium. In addition, they demonstrated adaptive production of pectinase in *Botrytis cinerea*.

A similar opportunity to examine such a relationship occurs in pea wilt (caused by *Fusarium oxysporum* f. *pisi*), in which the quality of the exudate from host roots is an important factor governing the resistance of pea varieties (Buxton 1957a, b). For example, pathogenic race 1 cannot grow in root exudate from the race 1-resistant pea variety wilt resistant Alaska, whereas race 2, which is pathogenic to Wilt-resistant Alaska, is stimulated by the exudate. However, after spores of race 1, obtained from genetically pure uninucleate parental material, were retained in Wilt-resistant Alaska root exudate for 14 days, they became pathogenic toward that variety. (Fig. 13).

This shows that a modification from race 1 to race 2 pathogenicity took place during the period of retention in the root exudate of the resistant variety. Moreover, the effect on race 1 was greater after it had been retained in exudate of 4 times the normal concentration. The fact that each aliquot of race 1 spores in exudate used in the experiments acted like race 2 after 14 days' retention seems to indicate that mutation could not be wholly responsible for this change. If mutation were assumed to have occurred, each aliquot would be expected to become a mixture of races 1 and 2. However, this did not occur, for plants inoculated with race 1 which had been kept in exudate all wilted without exception, and no controls wilted. The alternative explanation that the change was caused by enzymatic adaptation enabling race 1 to use, or alternatively to tolerate, some substance present in the exudate seems equally likely (Buxton, 1958). Changes of this type could result in acquired ability to tolerate and even make use of substances similar to the exudate that are present inside the host roots, available after penetration.

The results with both *Fusarium oxysporum* and *Phytophthora infestans* go some way toward explaining the sudden appearance of diseases in areas or among host varieties in which they have not previously been recorded. Such outbreaks of *Fusarium* diseases are common and there is a well-known pattern of the annual appearance of new races of the blight fungus as the season advances (Toxopeus, 1956). It would also be interesting to know whether losses of pathogenicity, well-known in fusaria in culture (Armstrong *et al.*, 1940) and common in several other fungi, are caused by a similar adaptive mechanism.

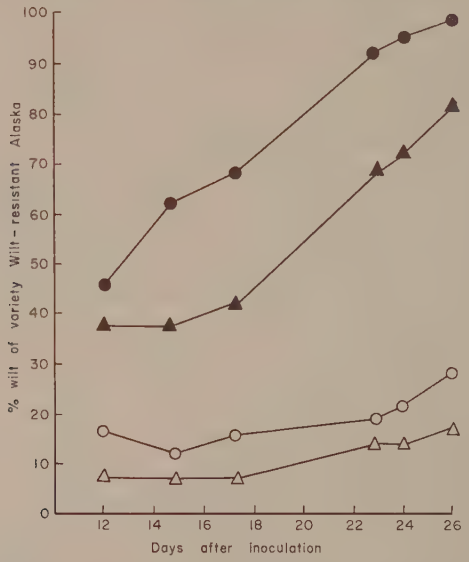


FIG. 13. Wilting caused by *Fusarium oxysporum* f. *pisi* race 1 after 14 days' retention in root exudate of pea variety wilt-resistant Alaska. ●: Race 1 retained in exudate at concentration 4 times normal. ▲: Race 1 retained in exudate of normal concentration. ○ Race 1 retained in sterile distilled water. △: Uninoculated plants.

Variations by adaptation, no less than other mechanisms of variation discussed in this review, have figured in the now rapidly growing recognition of the very great range of variability that fungi can draw on, and there seems little doubt that passage through a given host can alter the virulence of some pathogens. The question of how far the physiology of a fungus may be influenced by the host environment, and how permanent the resulting modifications are, can best be answered by making more studies of adaptation *in vitro*, but until more is known, caution is needed in interpreting its basic mechanism. It has, however, become evident that many of the problems of variation in pathogenic microorganisms are not easy to explain by any other mechanisms than adaptation, taking the definition in its general sense. While many adaptive changes are only temporary and are readily reversed, many may be permanent and hereditable, but even temporary changes would last long enough to cause damage to the host.

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CHAPTER 11

Genetics of Pathogenicity[1]

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I. INTRODUCTION

Knowledge of the inheritance of pathogenicity in phytopathogenic organisms is a product of our own time—principally of the second and third quarters of this century. This knowledge, however, could not have been acquired had it not been for the groundwork laid by earlier generations. In this respect several earlier lines of investigation are fundamental. The culturing of pure lines of fungi and other microorganisms, the understanding of the mechanism of transmission of hereditary qualities, and the development of the physiological race concept as applied to fungi were all fundamental to development of this field. Methods of culturing bacteria and fungi *in vitro* (developed by Koch, Brefeld, and others), investigations of host-pathogen relationships in the studies of Biffen on the inheritance of resistance in wheat to yellow rust (caused by *Puccinia glumarum*), and the erection of *formae speciales* of *Puccinia graminis* by Eriksson (1894) are specific milestones in this chain of development. The concept of the physiological race, however, is largely derived from the work of Barrus on bean anthracnose and of Stakman and his coworkers on stem rust of wheat.

Finally, the work of numerous mycologists and cytologists who studied the life cycles, morphology, and cytology of pathogenic organisms was essential to progress in genetic studies. On their work rest the methods of hybridization between pathogenic strains of fungi.

II. MUTATION

A. *Introductory*

Sexual processes leading to gene recombination do not occur in all microorganisms. Nevertheless, those organisms that do not appear to use the sexual method of transmission of hereditary characters have their own means of transmitting them and of producing variants. These means must be examined in any study of the inheritance of pathogenic properties. It is not possible to define mutation, for present purposes, in terms of mechanism. Therefore, in this discussion, mutation must be considered as any heritable change that can not be accounted for by other means such as recombination, heterokaryosis, transduction, or parasexual processes.

B. *Mutation in Asexual Organisms*

1. *Viruses*

Knowledge of the existence of variants in plant viruses dates back to McKinney's isolation (1926) of strains of tobacco mosaic virus that produce yellow spots instead of the ordinary mosaic symptoms. Kunkel (1947) stated that more than 400 strains of tobacco mosaic virus had been isolated or found to occur in nature. It is generally agreed that new virus strains are continually arising by mutation or a process related to it, but the rate of such changes differs in different viruses. The variants may differ from the parent strains by one or more of such characteristics as infectivity, longevity, point of thermal inactivation, optimum temperature for development, rate of movement in host tissues, type of lesion produced, host range, specificity to insect vectors, amino acid content, serological and immunological reactions, or other traits (Kunkel, 1947).

Variants are readily detected in the greenhouse and laboratory. Although they exist under natural conditions, their expression tends to be restricted by the greater invasiveness of highly adapted, common strains. In the laboratory, less infective variants may often be selected in a pure state from plants inoculated with a minimal infective dose. Dilution experiments have shown that the various characteristics of virus strains are heritable in the sense that they are perpetuated in serial transfers. However, mutability appears to be a characteristic of most strains. For tobacco mosaic virus, Kunkel (1940) estimated a mutation rate of about 0.5%. For the same virus, Gowen (1941) estimated the rate of mutation to the aucuba strain as 15.0×10^{-4} and the rate of the reverse mutation as 14.8×10^{-4}.

From the point of view of the inheritance of pathogenicity, mutation affecting host range is of particular importance. Kunkel (1940) states that the mutants of tobacco mosaic virus are not transmissible to plants immune from it and that they show immunological and serological relationship to tobacco mosaic virus. Within the limits of variability of a virus, the production of mutants will give opportunities for various host-virus associations. In host species with resistance genes operative against virus strains, these host-virus relationships are, in effect, host-gene : virus-strain relationships.

Some light may possibly be thrown on host-virus relationship by the remarkable recent investigations of Gierer and Schramm (1956), and Fraenkel-Conrat *et al.* (1957). Their studies have shown that the tobacco mosaic virus rod is made up of ribonucleic acid (RNA) surrounded by a covering layer of protein. The two were separated and tested separately for infectivity. For the strains tested, infectivity and specific symptom production are properties of the RNA. In "hybrids" produced by combining the RNA of one strain with the protein of another (Fraenkel-Conrat *et al.*, 1957) the host symptoms resulting from infection are those of the strain supplying the RNA. The two components, however, are apparently necessary for the normal functioning of a virus strain.

When progeny studies were made with virus taken from lesions produced by the hybrids, there was some evidence for the presence of mutant (or possibly segregant) strains.

2. *Bacteria*

A very extensive literature has grown up on mutability of bacteria, but mutation for pathogenicity in plant-pathogenic bacteria has been little studied.

Wellhausen (1937) showed that successive passage of strains of *Phytomonas stewartii* through highly resistant lines of maize resulted in increased virulence whereas passage through susceptible lines tended to reduce virulence. This work was confirmed, for the same organism, by Lincoln (1940), who reported that a study of single-cell colonies from old stock cultures showed that these are composed of many variants, presumably of mutational origin, differing in virulence. In a resistant host there is selection in favor of the virulent types. In susceptible hosts both virulent and avirulent types are propagated, and there is a tendency toward diminished virulence. Lincoln demonstrated further that virulent single-cell cultures also lose virulence by passage through a susceptible host but maintain their virulence in successive passage through a resistant host. As these cultures started from single cells, the variability was considered to be mutational in origin. For the characters studied, the mutants appeared to have about the same degree of stability as the parent strains. The rate of mutation of colony characteristics ranged from 1 in 20,000 to 1 in 80,000 cells.

Lincoln and Gowen (1942) studied the effect of X-ray irradiation on mutation in the same species of bacteria. The characters studied were color, surface appearance and size of colony, and pathogenicity. In a weakly pathogenic strain virulence was more frequently increased than decreased, whereas in a highly pathogenic strain a decrease in virulence was more frequent. They concluded that X-ray induced mutation differs from spontaneous mutation only by increased production of mutants. In both types, mutants appeared to be as stable as the parent form from which they were derived.

Ark (1946, 1951) showed that certain naphthalene compounds and uranium salts may act as mutagens on *Corynebacterium michiganense, Erwinia carotovora,* and *Xanthomonas juglandis.* These mutagens affected both cultural characters and pathogenicity. Mutations to higher and lower virulence were produced.

These studies demonstrate that mutation or some process akin to it is an important factor in bacterial variation for pathogenicity.

3. *Imperfect Fungi*

Most of the imperfect fungi are related to the Ascomycetes: both are haploid, the imperfect fungi entirely so, while most ascomycetous fungi are haploid during their period of growth and dikaryotic or diploid only in the reproductive stage. With respect to mutation, the chief difference between these two types of fungi is that in an ascomycetous fungus with a functioning perfect stage, mutant factors have an opportunity for forming recombinations which is denied to them in an imperfect fungus. Since the fungi are haploid, mutations for pathogenicity or other characters should be immediately expressed unless expression is inhibited by epistasis or influenced by modifying factors. If more than one genetic type of nucleus is present in a mycelium, the pathogenic effect may be the result of an unstable balance between the nuclei present in the heterokaryon as postulated by Hrushovetz (1957) for *Helminthosporium sativum.* Such mycelia should be amenable to selection of more or less vigorous pathogenic types (see Vol. II, Chapter 10). Although heterokaryosis may influence the expression of mutant factors, the initial source of variation is mutation.

Many ascomycetous fungi are highly mutable for cultural characteristics, the trend of mutation most frequently being from a conidial to a mycelial type, as described by Hansen (1938) for imperfect fungi.

There are fewer references in the literature to mutations for pathogenicity. A prerequisite for mutation for pathogenicity is the existence of lines of distinctive pathogenicity comparable to the physiologic races of the rusts. Though not always described as physiologic races, such lines have been found in many Ascomycetes or imperfect fungi related to them.

Any mutation for pathogenicity involves some change in the relationship between the pathogenic race and some host gene (or genes) concerned with host reaction. The work of Langford (1937) and Bailey (1950) has thrown some light on the genetics of host-pathogen relations of the tomato *(Lycopersicon esculentum)* and the leaf mold fungus, *Cladosporium fulvum.*

Langford (1937) showed that the Red Currant tomato *L. pimpinellifolium* carries a dominant gene, Cf_2*, on the fourth chromosome which confers immunity against races 1 and 2 of the leaf mold fungus. On the fifth chromosome is located an independently segregating dominant gene, Cf_3, which is hypostatic to the immunity gene and, in its absence, conditions resistance to these two races.

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* The genes are named according to the simplified designation of Rick and Butler (1956). Cf_1 was orginally designated as Cf_{sc}, Cf_2 as Cf_{p1}, and Cf_3 as Cf_{p2}.

By crosses with varieties of *L. esculentum,* these two genes were introduced into economically useful tomato varieties: Cf_2 into Vetomold and Cf_3 into V-121. A third dominant gene for resistance, designated by Langford as Cf_1, was present on the third chromosome of the variety Stirling Castle. In the variety V-473 this last gene was combined with gene Cf_2 to give a more broadly based resistance.

Bailey (1950) stated that in 1934 and 1935 only races 1 and 2 of *C. fulvum* were known to exist in Ontario. Gene Cf_2, contained in the greenhouse tomato variety Vetomold, was effective against these races. After the introduction of this variety, leaf mold disappeared wherever Vetomold was grown exclusively.

In 1939 leaf mold reappeared on Vetomold. Race studies showed that this leaf mold was a new race (race 5), which was like the formerly prevalent race 1, except that Vetomold was susceptible, that is to say, gene Cf_2 was ineffective against the new race.

Vetomold, now suffering severely from leaf mold infection caused by race 5 was replaced in 1941 by the variety V-121, which contained gene Cf_3, effective against race 5 as well as the formerly common races 1 and 2. After a period of freedom from mold, V-121 began to show sporadic infections. Race studies showed that these were caused by a new race (race 7) similar to race 5 except that V-121 was susceptible. Vetomold, which carried gene Cf_2, and Vineland Red Currant, which carried genes Cf_2 and Cf_3, were also susceptible to race 7. Race 7 was the only race isolated from 1947 to 1949 from commercial greenhouses in which these varieties had been grown.

Gene Cf_1, in the variety Stirling Castle, was effective against the originally common race 1 as well as the new races 5 and 7, but was ineffective against race 2. In 1940 a second race capable of attacking Stirling Castle was found, but only in trace amounts. This race, described as race 6, resembled race 7, except that the gene Cf_1 conditioned resistance to Stirling Castle and was ineffective against it. Later, considerable leaf-mold infection on this variety was found to be caused by yet another race, race 8, which was like race 5, except that Stirling Castle was susceptible as was V-473, which combined genes Cf_1 and Cf_2.

Finally, still another race (race 9) was isolated, presumably from plants of V-121 growing in greenhouses in which race 1 had been predominant. This new race was like 1, except that V-121 was susceptible instead of resistant.

Bailey (1950) advances strong arguments in favor of the hypothesis that the new races (5, 6, 7, 8, and 9) are mutants which may be traced back to race 1, the original wild type of *C. fulvum.* This presumed mutation process proceeded stepwise, each mutation overcoming the protective effect of a single host gene as indicated in Fig. 1. Thus, race 1 by two separate, single-step mutations gave rise to races 5 and 9; race 5 by two single-step mutations, to races 7 and 8; while race 7 by one such mutation produced 6. This latter race, which attacks all the sources of resistance, seems to be the end of this chainlike process.

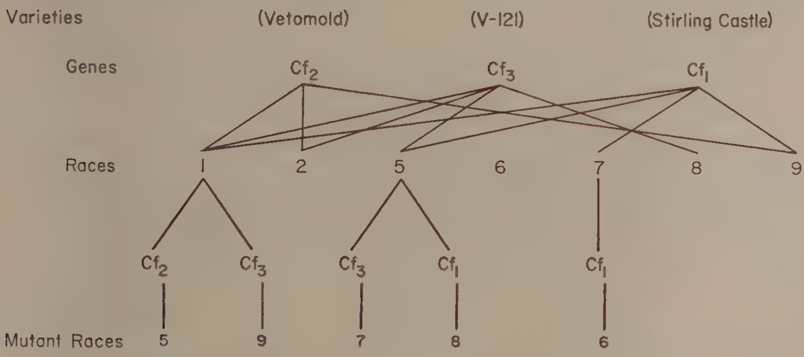

FIG. 1. Genes governing resistance to races of *Cladosporium fulvum* in certain tomato varieties and mutations in the fungus rendering ineffective the resistance conferred by certain genes.

Bailey's hypothesis of stepwise mutations has been strengthened by the work of Day (1957) who, by means of ultraviolet irradiation, induced a mutation of race 0 (Bailey's race 1) to race 2 (Bailey's race 5), thereby producing experimentally a mutation which Bailey had conjectured from circumstantial evidence. The fact that Day's mutation was induced in a red-pigmented culture eliminated contamination as a source of error.

C. *Mutation in Sexual Organisms*

1. *Phytophthora infestans*

In sexual organisms the added significance of mutation is that the mutant factors become subject to the process of recombination. However, in many fungi in which a perfect (sexual) stage is known, the occurrence of this stage is so rare that the sexual process is likely to play little part in variability. One such fungus is *Phytophthora infestans,* in which the sexual stage occurs infrequently. This organism provides one of the clearest examples in phytopathological literature of mutation as a means of pathogenic variation.

Giddings and Berg (1919) showed the existence of pathogenic strains. Isolates from potato were weakly pathogenic on tomato, whereas those from tomato attacked both potato and tomato vigorously.

The opportunity to demonstrate distinct races in the potato strain of *P. infestans* came with the incorporation in the potato of resistance genes from *Solanum demissum*. Black (1952a) showed that four of these resistance genes (R1, R2, R3, R4) conditioned resistance to races of potato late blight, each gene determining resistance to certain races. These genes, referred to as major genes, are inherited independently in simple Mendelian fashion.

The introduction of the *demissum* genes R1, R2, R3, and R4 into potato varieties separately and in all 14 possible combinations gave unique opportunities for demonstrating a relationship between the physiologic specialization of the pathogen and the genotype of the host. Helpful in establishing this relationship were several spontaneous mutations recorded by Black (1952b). All of these mutations showed an extension of pathogenic range obviously related to the genic constitution

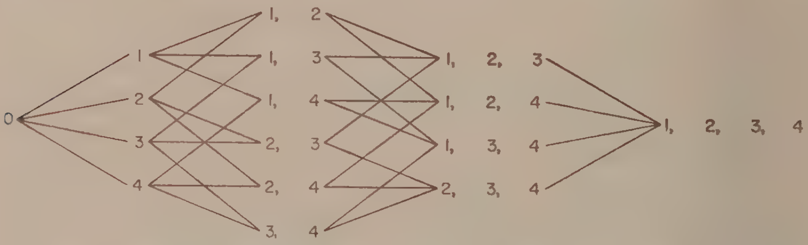

FIG. 2. Diagram illustrating how races of *P. infestans* can produce other races by single-step mutations. (From Black *et al.*, 1953.)

of the hosts. In the following itemization of the mutations, the R-factor constitution of the variety parasitized is given in parenthesis. Race A(r) → race D(R4); race B(R1) → race B2(R1, 4); race E(R1, 3) → race F(R1, 3, 4); race I(R3, 4) → race F(R1, 3, 4). It will be observed that all the mutations are single-step changes in each of which the pathogen gains an ability to overcome the resistance effect of a single R gene. Further evidence for single-step mutations was gained in a successful attempt to change race D(R4) to race C(R2, 4) by growing race D for four spore generations on detached leaves of a plant of genetic constitution R2.

Black concluded that "specialization proceeds along particular lines only and that these lines are determined by the genetic relationships of the hosts." This hypothesis led to the designation of each parasitic race by the resistance factors it can overcome: the common race, ineffective against all R factors, is designated as 0; other races as 1; 1,2; 1,2,3; 1,2,3,4; etc.

The diagram (Fig. 2) illustrates the mutational sequence by which races of *P. infestans* can produce other races by single-step mutations.

The fact that *P. infestans* adapts readily to sources of resistance has been established by many investigations. The mechanics of this adaptation are not entirely clear. Graham (1954, 1955) considers the fungus, as found in nature, to be heterokaryotic but maintains that zoospores are uninucleate. If they are uninucleate, the adaptation reported by De Bruyn (1951) and others in single-zoospore cultures is probably principally or entirely of mutational origin.

2. *The Rusts*

It has been shown by growing rust cultures in isolation for long periods that mutations for pathogenicity do not occur frequently. Stakman *et al.* (1930) reported a mutation in a culture of wheat stem rust only after it had been grown in pure culture for 13 years; Mehta (1940) reported that he had kept a race of wheat stem rust and two races of wheat leaf rust in culture for 100 uredial generations without the occurrence of a mutation; and Vasudeva *et al.* (1955) stated that some cultures had been kept for more than 300 generations without observable mutation for pathogenicity.

Recent work (Watson, 1957a) has demonstrated mutations for increased virulence in single-spore cultures of wheat stem rust under conditions of mass increase of spores on susceptible plants. Though heterokaryosis (see Vol. II, Chapter 10) is not here under discussion, several recent studies (Nelson and Wilcoxson, 1954; Nelson *et al.*, 1955; Nelson, 1956; Watson, 1957b) have shown that mutant factors may be given expression through nuclear exchange between physiologic races. Evidently mutation is a more important source of pathogenic variation than hitherto supposed and mutations, presumably mostly recessive, have, in heterokaryosis, a means of expression additional to segregation in the sexual stage. However, unless somatic segregation occurs, this means of expression should, theoretically, be less effective than the sexual stage in bringing mutations to light.

The function of the sexual stage of *Puccinia graminis* in giving expression to mutant factors has been described by Johnson and Newton (1938) who found that several characters mostly disadvantageous to the propagation of the organism occurred in the F_2, F_3, and F_4 generations of a cross between two physiologic races of wheat stem rust. These characters included decrease in vigor of sporulation, suppression in some cultures of pathogenicity at high temperatures, loss of ability to produce aecia, and the production of uredia and telia on barberry in some cultures which had lost the ability to produce aecia. In some rust cultures

(Johnson and Newton, 1943), inability to produce aecia or even pycniospores was a characteristic of about half of the sporidial infections and, therefore, was traceable to defects in half of the sporidia, suggesting a 2:2 ratio for that character. Perhaps the most unexpected characteristic observed in *Puccinia graminis* was a tendency of some of the aeciospores of a culture of wheat stem rust to produce abortive pycnia on wheat (Johnson and Green, 1954), a characteristic with obvious evolutionary implications.

Since all these abnormalities have been found in the progeny of apparently normal uredial cultures, rust cultures must quite commonly contain mutant factors that give rise to new characters. For the most part these are unfavorable to the organism but, conceivably, some may have evolutionary significance.

The production of mutations by artificial methods to improve our understanding of the mutational process has been unjustifiably neglected in rust studies. Flor (1956b) produced mutations from avirulence to virulence in the flax rust fungus, *Melampsora lini* ,by ultraviolet irradiation of a hybrid race known to be heterozygous for several factors. Since virulence is recessive, a dominant and a recessive gene were present at each heterozygous locus, with the result that the race was avirulent on the flax varieties affected by these loci. A single hit that altered a dominant gene might be expected to change a genotype from Aa to aa. In effect, three separate gene changes from dominant to recessive, each affecting the reaction of a single flax variety, appeared to have occurred as a result of the irradiation. Other changes appeared to involve three genes so closely linked that the mutational change affected all three simultaneously. Thus mutagenic agents, judiciously used, may be employed to bring about results desired by the investigator. The total number of infections caused by the irradiated spores in relation to the number of infections displaying mutant properties is a useful measure of the relative mutation rates of genes that condition pathogenicity.

3. *The Smuts*

When cultural studies were undertaken with haploid lines developing from sporidia of germinated teliospores, it became apparent that mutation for cultural characters was frequent in some species. Because these haploid lines are nonpathogenic, direct observation of mutation for pathogenicity was not possible. There is, however, no *a priori* reason for supposing that mutations directly affecting pathogenicity do not occur in the haplophase. In fact, circumstantial evidence that they do occur is available from the work on *Ustilago maydis (U. zeae)* by Christensen and Stakman (1926) and Stakman *et al.* (1929), and it was shown by Stakman *et al.* (1933) and Rowell and DeVay (1954) that some of these mutants may exceed the original line in virulence. Because mutations for pathogenicity must be detected indirectly in dikaryotic combinations of mutant lines, there is little knowledge of their frequency of occurrence.

III. HYBRIDIZATION

A. *The Smuts*

1. *Pathogenic Relationships in the Haploid and Dikaryotic Stages*

Although there is a great diversity of cytologic behavior in the smuts, a common pattern may be observed in the species that have been studied. The pathogenic mycelium of a smut is dikaryotic. At the completion of pathogenic development the conjugate nuclei fuse in the young teliospore.* Since the teliospore is uninucleate (diploid), it might be thought that the diploid nuclear condition governs the change from the vegetative, that is, the pathogenic stage to the reproductive, or spore-forming stage. That this is not so is shown by the fact that the teliospores are already in process of formation, while the smut is still in the binucleate condition. In the germination of the teliospore, meiosis occurs with the production of four or, in some genera, eight nuclei in the promycelium. Further development in the haploid stage may be sporidial or mycelial, depending on the species, but is, almost universally, saprophytic. Before a pathogenic relationship can be established with the natural host of a smut, sporidia or mycelia of compatible mating types must fuse to reestablish the dikaryophase.

In many smut species an immense amount of variation has been observed in the haplophase. Though much of this variation is of interest genetically, it bears little relation to pathogenicity, because the haplophase is the nonpathogenic phase of the smut life cycle. The occurrence in the haplophase of mutations for pathogenicity that later gain expression in the dikaryophase has been mentioned earlier.

2. *Sexual Phenomena*

The smuts are suitable material for genetic studies because (1) meiosis occurs in the germination of the teliospore, (2) the products of meiosis, usually in the form of sporidia, can be transplanted to artificial media for the study of mating reactions and cultural characteristics, (3) the haploid cultures thus obtained can be introduced into the host plant in sexually compatible combinations and so permit a study of the inheritance of various characters in the dikaryophase of the smut.

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* In conformity with the practice of Fischer and Holton (1957) the term teliospore is used in place of chlamydospore because functionally the so-called chlamydospore of the smuts is identical with the teliospore of the rusts.

The basic investigations that contributed the knowledge necessary to the undertaking of genetic studies extended over the period of about half a century from DeBary's (1866) observation of fusion between sporidia to Kniep's (1919) demonstration that cell fusion and nuclear association take place only in certain sporidial combinations. Historical reviews of these and intervening studies are given by Hanna (1929) and Fischer and Holton (1957). Kniep (1919) found that cell fusion and nuclear association take place only in certain sporidial combinations. The sporidia were of two mating types determined by segregation of sex factors during the germination of the teliospore. Since Kniep's discovery, his methods of study have been applied to many species of smuts. Bipolar sexuality (two mating types) conditioned by a single pair of alleles has been found to prevail generally in the cereal smuts. Multipolar sexuality conditioned by two or more pairs of alleles has been reported in several smuts. Reviews of mating-type behavior in the smuts may be found in Ainsworth and Sampson (1950), Whitehouse (1951), and Fischer and Holton (1957).

Probably most inheritance of smut characteristics is dependent on regular meiotic behavior in teliospore germination. Deviations from ordinary meiotic behavior have been reported in several smut species. Holton (1952) reported homothallism in the grass smut (*Tilletia elymi*) and Siang (1954) recorded similar behavior in another smut of grasses, *Tilletia cerebrina*. In both instances the primary sporidia contained several nuclei which had evidently migrated from the promycelium. These are probably to be regarded as cases of secondary homothallism, perhaps not a highly fixed condition, as Siang (1954) reports uninucleate sporidia occasionally arising from his homothallic lines.

The most extensively studied "homothallic" condition in the smuts is that of the well-known solopathogenic lines reported in *Ustilago maydis* by Stakman and Christensen (1927) and further studied by Christensen (1929, 1931), Chilton (1943), Rowell and DeVay (1954), and others. These lines, usually originating from single sporidia, grow on artificial media where they cannot be distinguished from haploid lines. Unlike haploid lines, they singly cause infection and gall formation in the host. The stability of the homothallic condition seems to vary considerably, some lines being rather highly fixed, others now and then giving rise to haploid lines (Chilton, 1943). The derivation of haploid lines of ordinary mating types from solopathogenic lines either by natural segregation processes or, as demonstrated by Rowell (1955), by somatic segregation following alpha radiation, would suggest that Chilton (1943) is correct in his assumption that these lines are diploid due to failure of normal disjunction of chromosomes.

3. *Pathogenicity Ratios*

Combinations occur among the haploid elements of a single germinating teliospore, and the pathogenicity ratios in the dikaryophase are dependent on the arrangement of pathogenicity and mating type factors brought about by first and second division segregations. First division segregation for mating type gives + + — — or — — + +; second division segregation gives + — + —, or — + — +, or — + + —, or + — — +. These are the only possible arrangements unless the disposition of the meiotic products is disturbed by the "slipping past" of nuclei in the promycelium. Pathogenicity combinations depend on the relation of the segregation of pathogenicity factors to that of mating type factors. First division segregation for *both* mating type and pathogenicity results in a 4:0 ratio

$$\frac{+\;+\;-\;-}{\mathrm{A\;A\;a\;a}} \rightarrow \mathrm{Aa,\ Aa,\ Aa,\ Aa}$$

First division segregation for mating type and second division segregation for pathogenicity, or vice versa, is independent segregation and gives a 1:2:1 or, with dominance, a 3:1 ratio, e.g.,

$$\frac{+\;+\;-\;-}{\mathrm{A\;a\;A\;a}} \rightarrow \mathrm{AA,\ Aa,\ Aa,\ aa,\ or}\ \frac{+\;-\;+\;-}{\mathrm{A\;A\;a\;a}} \rightarrow \mathrm{AA,\ Aa,\ Aa,\ aa}$$

With second division segregation for both mating type and pathogenicity, the diplophase pathogenicity ratios may be 4:0 or 1:2:1, depending on the arrangement of factors for pathogenicity in the promycelium:

$$\frac{+\;-\;+\;-}{\mathrm{A\;a\;A\;a}} \rightarrow \mathrm{Aa,\ Aa,\ Aa,\ Aa};\ \frac{+\;-\;+\;-}{\mathrm{a\;A\;a\;A}} \rightarrow \mathrm{Aa,\ Aa,\ Aa,\ Aa};$$

$$\frac{+\;-\;+\;-}{\mathrm{A\;a\;a\;A}} \rightarrow \mathrm{Aa,\ AA,\ aa,\ Aa};\ \frac{+\;-\;+\;-}{\mathrm{a\;A\;A\;a}} \rightarrow \mathrm{Aa,\ aa,\ AA,\ Aa}$$

The result is the same if the pathogenicity factors are held constant and the mating type factors arranged in the four possible ways.

With two mating types, + and —, but two pairs of alleles governing pathogenicity, the pathogenicity ratios in the dikaryophase may become somewhat more complex. Ratios of 4:0, 1:2:1, and 1:1:1:1 become possible, as is shown in the following listing in which the four second division and two first division mating type arrangements are matched with three of the various possible gametic distributions of the pathogenicity factors. Assuming complete dominance, some 1:2:1 ratios would become 3:1 ratios. Ratios of 2:2, frequently reported in smut literature, may be derived from the 1:1:1:1 ratios on the assumption that the A and B loci affect the same characters but in such a way that three dominant genes, such as AABb, produce one effect and three recessive genes, such as aaBb, another.

1. AB AB ab ab AB in coupling (Ditype)

$$\left.\begin{array}{cccc} + & - & + & - \\ - & + & - & + \\ + & - & - & + \\ - & + & + & - \end{array}\right\} \text{AABB, AaBb, AaBb, aabb} - 1:2:1$$

$$\left.\begin{array}{cccc} + & + & - & - \\ - & - & + & + \end{array}\right\} \text{AaBb, AaBb, AaBb, AaBb} - 4:0$$

2. Ab Ab aB aB AB in repulsion (Ditype)

$$\left.\begin{array}{cccc} + & - & + & - \\ - & + & - & + \\ + & - & - & + \\ - & + & + & - \end{array}\right\} \text{AAbb, AaBb, AaBb, aaBB} - 1:2:1$$

$$\left.\begin{array}{cccc} + & + & - & - \\ - & - & + & + \end{array}\right\} \text{AaBb, AaBb, AaBb, AaBb} - 4:0$$

3. AB Ab aB ab First division segregation for A, second division for B (Tetratype)

$$\left.\begin{array}{cccc} + & - & + & - \\ - & + & - & + \end{array}\right\} \text{AABb, AaBb, AaBb, aaBb} - 1:2:1$$

$$\begin{array}{cccc} + & - & - & + \end{array} \quad \text{AABb, AaBB, Aabb, aaBb} - 1:1:1:1$$

$$\begin{array}{cccc} - & + & + & - \end{array} \quad \text{AABb, Aabb, AaBB, aaBb} - 1:1:1:1$$

$$\left.\begin{array}{cccc} + & + & - & - \\ - & - & + & + \end{array}\right\} \text{AaBB, AaBb, AaBb, Aabb} - 1:2:1$$

The order of the gametic genotypes in the promycelium, such as AB, Ab, aB, ab, or AB, ab, Ab, aB, may affect ratios but, whatever the order, the ratios produced will be one or another of the above. Phenotypic ratios may, of course, be expected to show modifications resulting from genic interaction, epistasis, etc.

The above patterns of distribution may be seen in smut literature in analyses of segregations of mating type factors or of recombinations of pathogenicity factors. Nicolaisen (1934), in a study of *Ustilago avenae,* found all six arrangements of mating type factors in his analyses of promycelia of 73 teliospores—first division reduction occurring 29 times and second division 44 times. Halisky (1956) records for the same species that seven of 22 germinating teliospores showed reduction in the first division and 15 in the second. In pathogenicity studies of progenies from these spores, Halisky obtained pathogenicity ratios of 4 : 0, 3 : 1, and 2:2. The same pathogenicity ratios appear in the selfing studies of Nicolaisen.

Factors for mating type and cultural characteristics are the commonly cited examples of segregation in teliospore germination. However, some of the best examples of the inheritance of characteristics expressed in haploid lines are those showing the inheritance of factors for growth deficiencies induced by ultraviolet radiation (Perkins, 1949). Perkins studied the segregation obtained from single zygotes derived from crosses in which two or more loci were involved and recovered all possible combinations of mutant factors. For example, the cross Isoleucineless-Methionineless (I — A + M —) × Adenineless-Methionineless (I + A — M —) produced, from a single teliospore, the parental combinations plus the two recombinants I + A + M — and I — A — M —, which expresses a 1 : 1 : 1 : 1 ratio.

4. *Concept of Hybridization*

Hybridization is a term which has been used in different senses by different individuals. Kniep regarded fusion of sporidia as a criterion of hybridization. This is using the term in its broadest sense. To obtain genetic information it is necessary not only that the sporidia fuse but also that the nuclei of the sporidia establish the binucleate, conjugate relationship; that they fuse in the teliospore with subsequent reduction divsion; and finally, that the products of reduction division, the sporidia of the F_1 teliospore, unite to produce a new pathogenic generation. It is hybridization in this narrower sense that is of chief interest in this discussion.

Fortunately, hybridization of this type is not entirely confined to intraspecific crosses. Interspecific, and occasionally intergeneric crosses, produce F_1 teliospores which are sufficiently normal in their meiotic processes to permit a study of genetic phenomena in the F_2 generation. Interspecific crosses are of particular interest not only because they enable a study of the genetics of characters on which species are determined but also because they throw light on the origination of morphologic and pathogenic characteristics that are important in the evolutionary process.

5. *Inheritance of Sorus Type*

Soral characteristics are pathogenic properties and are important criteria for the distinction of species. Because these characters differ between species rather than within species, it is from interspecific rather than intraspecific crosses that information may be gained on their inheritance. Smut literature describes many crosses between the loose smut of oats, *Ustilago avenae,* and the covered smut, *U. kolleri.* The loose smut type has generally been found to be dominant (Hanna and Popp, 1930; Holton, 1931; Cherewick, 1958). Holton's finding (1932) that intraspecific combinations in *U. avenae* produced both the loose and the covered types is not surprising in view of the interfertility of the species and the dominance of the loose smut type. Intraspecific combinations in *U. kolleri* produced only covered smut, the recessive character. Dominance of the loose smut type of *U. avenae* was shown not only in crosses with *U. kolleri* but also in crosses with the more distantly related *U. perennans* (Fischer and Holton, 1941).

One deviation from the normal, in loose smut, that has been studied genetically is the indurate type of smutted panicle in which the spores are held in compact sori despite the destruction of the glumes. Holton (1941) crossed the normal, powdery type of smut with the indurate type. The powdery sorus (P) was dominant to the indurate (p), the heterozygote (Pp) being powdery. Matings of the sporidial lines of five F_1 teliospores showed two types of segregation—4 : 0 and 3 : 1. In three of the spores there was independent segregation of factors for mating type and sorus type which resulted in a ratio of 3 powdery to 1 indurate. In two spores segregation of factors for mating type and sorus type occurred in the same nuclear division and, therefore, all the sori were the powdery, 4 : 0 ratio.

Ustilago hordei, the covered smut of barley, crosses readily with *U. nigra (U. medians),* the false loose smut. Allison (1937) crossed the two species and found the smutted head type in F_1 to be intermediate. In F_2 both parental types appeared as well as an intermediate type. Bever (1945) obtained similar results. It may be concluded that intermediate smut types do arise from hybridization, natural or artificial, between these species.

Another group of smut species sufficiently closely related to permit crossing and genetic studies are the sorghum smuts, *Sphacelotheca sorghi, S. cruenta, and Sorosporium reilianum (Sphacelotheca reiliana).* Rodenhiser (1934) crossed the loose kernel smut, *S. cruenta,* with the covered kernel smut, *S. sorghi,* and found the loose type dominant. Segregating progeny contained both types and also intermediate types. Vaheeduddin (1942) crossed *S. cruenta* with the head smut of corn, *Sorosporium reilianum,* and reported that different monosporidial combinations produced sori differing in shape and size. Some resembled those of *S. cruenta,* some those of *S. reilianum,* and still others resembled sori of the long smut of sorghum, *Tolyposporium filiferum.* The same three types reappeared in the F_2 generation.

These few examples are sufficient to show that much hybridization occurs between smuts which are genetically related although sufficiently distinctive in appearance to be classified as separate species. There is a strong tendency, especially in the cereal smuts, for the loose smut habit to be dominant over the covered. Some smuts of intermediate type may have originated as hybrids. Although there is no proof, there is circumstantial evidence, particularly in the work of Ruttle (1934), that the false loose smut of barley, *U. nigra*, may have arisen from hybridization between the loose smut, *U. nuda*, and the covered smut, *U. hordei*.

6. *Hybridization in Relation to Pathogenicity*

In the smuts, as in the rusts, the dikaryotic physiologic race is basic to any study of pathogenicity. The concept of the physiologic race was established in studies on long-cycled rusts, particularly *Puccinia graminis*. In these rusts, uredial clones, which could be kept in culture indefinitely, became the criteria of specialization, irrespective of whether they were pathogenically homozygous or heterozygous. In the smuts, in which no such clones exist, a culture can not be considered a physiologic race unless it is stable from one generation to the next for those pathogenic characteristics with which the investigator is concerned.

It was natural that physiologic races should first be determined in the cereal smuts, not only because of their economic importance but because the presence in cereals of a large number of closely related but genetically distinct host varieties provides the cereal smuts with opportunities to display any inherent pathogenic differences. When physiologic specialization in the cereal smuts became appreciated, hybridization studies were quickly undertaken in the smuts of cereals, corn, and sorghum. These studies, too numerous to be reviewed here, showed that, with rare exceptions, host plants could not be infected with smut lines of monosporidial origin. The smuts were heterothallic, and two monosporidial lines of compatible mating types were necessary to bring about infection.

The fact that the sporidia produced by the germinating teliospore will produce cultures on artificial media was of great advantage to genetic studies. The homozygosity or heterozygosity of an individual teliospore could be determined, and it, rather than the mass culture of a physiologic race, became the basic unit in most genetic studies.

Information on the inheritance of pathogenicity may be obtained from (1) hybridization between species (interspecific crossing) and (2) hybridization between races within species (intraspecific crossing). Few investigators have ventured on any genetic analysis of their results because of the complexity of the inheritance of pathogenicity.

a. *Interspecific versus Intraspecific Hybridization.* Studies on interspecific hybridization have shown the existence of sterility barriers. Their effectiveness varies greatly in different interspecific combinations. Most evidence for interspecific sterility comes from *in vitro* experiments: failure of development of sporidia on promycelia of F_1 teliospores, as observed by Holton (1932) for hybrids of *Ustilago avenae* and *U. kolleri,* and by Rodenhiser (1934) for hybrids of *Sphacelotheca sorghi* and *S. cruenta;* low viability of the sporidia produced as reported by Martin (1943) for hybrids between *Sorosporium syntherismae* and *Sphacelotheca panici-miliacei;* and lysis of promycelia and sporidia as in F_1 hybrid spores of *Ustilago hordei* and *U. bullata* (Fischer, 1951). However, some of these sterility barriers, such as lysis (Fischer, 1940, 1951), are functional against saprophytic growth and not against pathogenicity. Despite sterility barriers, hybridization can occur between species of smuts with a common host plant. Effective and possibly complete sterility barriers may exist between species that lack a common host. Allison (1937) found that the barley smuts *Ustilago hordei* and *U. nigra* would hybridize with the oat smuts *U. avenae* and *U. kolleri* and the wheat smut *U. tritici* to the extent of sporidial fusions and the initiation of a dikaryophase but not to the extent of the production of hybrid spores or even of smut mycelia in the plants; Fischer (1953) obtained similar results in his attempts to hybridize the two above-mentioned barley smuts with the grass smut *U. striiformis hordei.*

Interspecific hybridization is potentially an important source of new pathogenic types of smut, but no generalization concerning the pathogenic characteristics* of species hybrids is possible. In the F_1 dikaryophyte various degrees of intermediacy are commonly expressed. In crosses between *Sphacelotheca sorghi* and *S. cruenta* Rodenhiser (1934) found that on varieties of sorghum resistant to one species and susceptible to the other, most of the interspecific hybrids were intermediate in infectivity; but the same held true for intraspecific hybrids. On the variety Reed kafir, which was attacked by both parent species, he found the interspecific combinations less infective than the parent lines, whereas intraspecific combinations of lines of *S. sorghi* were more infective than parent lines.

In crosses between *U. hordei* and *U. nigra,* Bever (1945) found that *U. hordei* race 6, which did not attack the varieties Nepal and Himalaya, when crossed with *U. nigra* race 6, which attacked them moderately, produced an F_1 hybrid which attacked both varieties lightly.

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* Although soral characteristics are one means of measuring pathogenicity, the usual means, and the one here under consideration, is infectivity, generally expressed as the percentage of inoculated plants showing infection.

Although various degrees of intermediacy are probably the most common pathogenic traits of F_1 species hybrids, complete dominance of one specific trait over another does occur (Allison, 1937). The inability of *U. hordei* to attack Peatland was dominant in F_1 over the ability of *U. nigra* to attack that variety.

Infectivity of F_2 and later generations of crosses is inherited independently of such other traits as soral characters, spore-wall markings, and teliospore color. The various infectivity characteristics of one species may be combined in many ways with those of another to produce a new dikaryophyte pathogenically different from those lines of the parent species that entered into the cross. Since this conclusion applies equally to intraspecific crosses, there seems to be no difference in principle between inter- and intraspecific hybridization apart from the existence in the former of various degrees of intersterility and the production through interspecific crosses of soral and gross morphological characters that would not be expected from intraspecific crosses. For the examination of the genetics of infectivity, the two types of hybridization may, therefore, be considered together.

b. *Genetics of the Inheritance of Pathogenicity.* A few representative studies will be examined briefly to illustrate the infectivity of parent lines and their hybrid progeny and, where possible, to interpret pathogenic behavior genetically.

The oat smuts, *Ustilago avenae* and *U. kolleri,* have been studied at least as thoroughly as any other smuts. The work of Nicolaisen (1934) provides a valid example of the inheritance of infectivity in selfing and crossing studies. He performed matings among sporidial lines of single teliospores (here referred to as selfing) and matings among sporidial lines of two teliospores (referred to as crosses). He concluded (1) that most teliospores are heterozygous for degree of pathogenicity on one or other of the test hosts, (2) that pathogenicity is dominant on the variety Gopher but recessive on von Lochow. Pathogenicity was dominant in some cases and recessive in others on the variety Lischower. Thus, collections of *U. avenae* are populations of biotypes of different virulence, and the proportion of virulent types differs in different populations.

The tabulation, taken from Holton (1936), shows how readily the range of pathogenicity in smuts may be broadened by crossing two lines of smut of restricted pathogenicity. (See next page.)

Some of the hybrid recombinants were pathogenic to both Gothland and Monarch. Smut was selected from Gothland in F_2, and the selective effect on genotypes virulent to that variety is apparent in the high percentage of infection in F_3. A probable genetic explanation of the expanded range of infectivity is that one pair of alleles governs infectivity on Gothland, another on Monarch; heterozygotes would have some virulence on both varieties.

Holton (1941) made ingenious use of his buff smut, which had arisen by mutation in *U. kolleri*, to study the inheritance of pathogenicity. He obtained hybrid teliospores by inoculating susceptible oats with paired monosporidial cultures of the buff smut and *U. avenae*. The F_1 teliospores were used to inoculate differential varieties on which F_2 teliospores were produced. Since buff color is a recessive character, buff F_2 segregates were homozygous for color. He could, therefore, obtain new buff races by selecting buff spores from host varieties immune from the buff parent but susceptible to the *U. avenae* or *U. kolleri* parents. In this way five buff races were produced. This was possible because factors for spore color and pathogenicity were inherited independently. Of these races buff hybrid race 5 combined the most virulent characteristics of the two parent races employed in the cross from which it arose. Interestingly, buff hybrid race 6 was virulent to Red Rustproof C. I. 1355, which was not attacked by either of the parent lines.

PERCENTAGE OF INFECTION

	Parents		F_2	F_3[a]
	U. kolleri	*U. avenae*		
Gothland	0.0	100.0	22.0	92.0
Monarch	99.0	0.0	21.0	45.0

[a] F_3 selected from Gothland.

One of the few attempts to analyze factorially the inheritance of pathogenicity is that of Halisky (1956), who crossed races 5 and 6, 5 and 7, and 6 and 7 of *Ustilago avenae* and studied infectivity in the F_1, F_2, and F_3 generations on the differential hosts. Each cross was made by mating the eight sporidial lines from two teliospores (one of each race) in their eight compatible combinations, so that eight F_1 hybrids were obtained from the two teliospores.

The most clear-cut inheritance was that expressed on the variety Camas. Race 5 (avirulent) × race 6 (avirulent) gave avirulence in F_1 and in F_2. Thus, these two races were genotypically identical for infection characteristics on this host.

In the cross race 5 (avirulent on Camas) × race 7 (virulent) the F_1 was almost avirulent, i.e., pathogenicity was almost suppressed, indicating near-complete dominance of avirulence. The F_2 from three of the F_1 spores analyzed showed a 3 : 1 avirulent : virulent ratio, indicating monogenic inheritance. The F_2 from three other spores showed a 4 : 0

avirulent reaction. Although Halisky has a different interpretation, virulence is evidently recessive. The F_1 genotype can be designated Cc, C being derived from the avirulent race 5 and c from the virulent race 7. Segregation in F_2 is either 1CC: 2Cc; 1cc (giving a 3 : 1 ratio) or Cc: Cc: Cc: Cc (giving a 4 : 0 ratio). Twelve cultures, derived from three F_2 spores, were studied in F_3. It is worth noting that in all the heterozygous (Cc) lines there was a marked increase in pathogenicity in F_3 over that expressed in F_2. In these cultures the ½ Cc genotypes would have a slight survival value, whereas the ¼ cc genotypes would enjoy a high selective advantage. There was obviously a rigorous selection against the CC genotypes.

In the cross, race 6 (avirulent on Camas) × race 7 (virulent), there was complete dominance of avirulence, (i.e., 0 infection), in F_1. The F_2 generation showed a 3 : 1 ratio of avirulence to virulence, again indicating monogenic inheritance. Sixteen cultures derived from four F_2 spores were studied in F_3 with results similar to those mentioned above for the cross, race 5 × race 7, except that the increase in virulence in cultures derived from the heterozygotes was less pronounced. The same rigorous selection against the CC genotype was shown by the fact that four cultures from that genotype produced no infection on Camas.

On the variety Monarch, pathogenicity was inherited quite independently of that on Camas but, in principle, the inheritance is similar, monogenic with pathogenicity recessive and both 3 : 1 and 4 : 0 ratios in F_2. In F_3 a very marked increase occurred in pathogenicity through the selfing of heterozygous cultures. In 8 heterozygous cultures of the cross, race 5 × race 6, the average infection was about 7% in F_2 but rose to about 23% in F_3, an indication of the selective effect on the more virulent homozygous recessive types.

Any account of the inheritance of pathogenicity in the smuts would be deficient without some reference to hybridization in *Tilletia caries* and *T. foetida.* Flor (1932) showed that these species were heterothallic and Becker (1936) concluded from matings within *T. caries* that virulence might be either recessive or intermediate. Most of the evidence available, however, comes from the work of Holton, who studied interspecific and intraspecific hybridization in these smuts. Although his experiments have yielded much information on the pathogenic characteristics of the parent strains and their hybrid combinations, genetic interpretation is largely a matter for conjecture. Holton (1942) reports on studies of 50 hybrids, 23 from crosses of *T. caries* and *T. foetida,* and 27 from interracial combinations within the species.

Typical results of four crosses between *T. foetida* and *T. caries* in the F_2 and later generations of interspecific hybrids are shown in Table I.

Despite the fact that the same sporidial line of *T. foetida* was used in all four crosses there are at least three different pathogenic types present. Hybrid No. 35 resembles closely the *T. foetida* parent race. Hybrid No. 38 differs from both parents, whereas hybrid No. 39 combines the pathogenicity of both. From F_2 to F_5 hybrid No. 40 resembled hybrid No. 38, but in F_6 there is evidence for the selection of a type resembling hybrid No. 35. Since the genic contribution of spore L56 to all four

TABLE I

PATHOGENICITY OF INTERSPECIES HYBRIDS BETWEEN *Tilletia levis* (*T. foetida*) AND *T. tritici* (*T. caries*)[a]

Hybrid L-8 × T-9

Parent or hybrid no.	Pedigree	Inoculum		Smut in	
		Source	Generation	Oro (%)	Hohenheimer (%)
L-8	*T. foetida*	Oro		85	0
T-9	*T. caries*	Hohenheimer		0	30
35	L 56-1 × T 157-1[b]	Hard Federation	F_2	12	0
		Oro	F_3	70	0
		Oro	F_4	84	0
		Oro	F_5	77	0
		Oro	F_6	85	0
38	L 56-1 × T 157-4	Hard Federation	F_2	3	1
		Oro	F_3	1	0
		Hybrid 128	F_4	1	0
		Hybrid 128	F_5	1	0
		Hybrid 128	F_6	1	0
39	L 56-1 × T 157-5	Hard Federation	F_2	33	21
		Oro	F_3	30	27
		Hohenheimer	F_3	27	33
		Oro	F_4	24	22
		Hohenheimer	F_4	28	32
		Oro	F_5	28	26
		Hohenheimer	F_5	30	35
		Oro	F_6	84	20
		Hohenheimer	F_6	72	68
40	L 56-1 × 157-8	Hard Federation	F_2	10	0
		Oro	F_3	1	4
		Hybrid 128	F_4	7	0
		Hybrid 128	F_5	9	2
		Hybrid 128	F_6	64	5

[a] From Holton, 1942.

[b] *T. levis* parent, teliospore 56, sporidium 1, crossed with *T. tritici* parent, teliospore 157, sporidium 1.

crosses should have been the same, it seems obvious that any initial genetic differences in the F_1 hybrids were due to spore T157. Superimposed on these differences, however, are the important selective effects in later generations. In cross 35 selection on the variety Oro raised the percentage of infection from 12% in F_2 to 85% in F_6. Similarly, in cross 39, selection on Oro and Hohenheimer had raised the infectivity on both varieties to high level in F_6.

TABLE II

PATHOGENICITY OF INTER-RACE HYBRIDS OF *Tilletia tritici* (*T. caries*)[a]

Hybrid T-8 × T-9

Parent or hybrid no.	Pedigree	Inoculum		Smut in		
		Source	Generation	Albit (%)	Hohenheimer (%)	Hussar[c] × Hohenheimer (%)
T-8		Albit		89	0	0
T-9		Hohenheimer		0	30	0
50	T 60-1 × T 157-2[b]	Hindi	F_1	12	28	3
		Hussar × Hohenheimer	F_2	—	37	8
		Hussar × Hohenheimer	F_3	—	35	16
		Hussar × Hohenheimer	F_4	19	74	29
		Hohenheimer	F_2	20	37	4
		Hohenheimer	F_3	—	31	1
		Hohenheimer	F_4	13	62	2
		Hybrid 128	F_2	13	0	0
		Albit	F_2	83	0	0
52	T 60-1 × T 157-4	Hindi	F_1	8	10	3
		Hussar × Hohenheimer	F_2	—	27	6
		Hussar × Hohenheimer	F_3	—	50	18
		Hussar × Hohenheimer	F_4	71	77	36
53	T 60-1 × T 157-5	Hindi	F_1	3	1	0
		Hybrid 128	F_2	1	0	0
		Hybrid 128	F_3	—	1	0
		Hybrid 128	F_4	1	1	0

[a] From Holton, 1942.

[b] T-8 parent, teliospore 60, sporidium 1, crossed with T-9 parent, teliospore 157, sporidium 2.

[c] Hussar is susceptible to race 8 but immune from race 9.

In the three crosses between races 8 and 9 of *T. caries* (Table II) the combined virulence of the two parent races on Albit and Hohenheimer is evidenced by crosses 50 and 52, but the least virulent characteristics of both parents appear combined in cross 53. Transgressive segregation occurs in the first two crosses with respect to the variety Hussar × Hohenheimer, which is moderately attacked by much of the progeny, although it is attacked by neither parent race. The effect of host selection of virulent genotypes is clear especially with respect to Hussar × Hohenheimer. Selection on this host raised the percentage of infection in cross 50 from 3% in F_2 to 29% in F_4 and in cross 52 from 3% in F_1 to 36% in F_4.

Neither race 8 of *T. foetida* (Table I) nor race 8 of *T. caries* (Table II) has any pathogenicity on Hohenheimer. Clearly, in the above crosses pathogenicity to Hohenheimer was derived from *T. caries* race 9, and, in fact, from the same teliospore of it, teliospore 157. Three of the monosporidial lines from this spore were used in the interspecific and the intraspecific crosses. If a given sporidial line contributed pathogenicity in one cross it would be expected to do so in another, but this was not the case. In each instance in which a sporidial line was used in an interspecific and an intraspecific cross the results were different. Sporidial lines 1 and 4 contributed pathogenicity only in the intraspecific crosses; sporidial line 5, only in the interspecific cross. Each F_1 heterozygote derived from a cross with spore 157 should have contained the gene or genes for virulence on Hohenheimer. If this were so, the presence in subsequent generations of the virulence genes in some crosses and their absence in other crosses must be due to selection. If the F_1 teliospores of a cross should give rise to only a few infections, the genotypic composition of the F_2 generation would be limited to a few or perhaps only a single chance genotype. Possibly the absence of virulence to Hohenheimer in crosses where virulence is expected may be a consequence of such selection.

In explaining the transgressive segregation on the variety Hussar × Hohenheimer, which is immune from both parent races (races 8 and 9 of *T. caries*) but susceptible to some of the progeny of crosses 50 and 52 (Table II), it is necessary to state that Hussar is susceptible to race 8 but immune from race 9. The F_1, therefore, contains factors for pathogenicity to both Hussar and Hohenheimer. Selection on Hussar × Hohenheimer from F_2 onward in crosses 50 and 52 would tend to build up an increasing concentration of those genotypes containing virulence factors for Hussar, derived from the race 8 parent, and virulence factors for Hohenheimer derived from the race 9 parent.

The importance of selection pressure, so evident in the examples just cited, can scarcely be overemphasized in the smuts, which pass through a sexual cycle in each spore generation. Holton (1947) has emphasized the importance of varietal reaction in the origination of races of various degrees of virulence and has stated that highly susceptible varieties, such as Hybrid 128, promote the establishment of wheat bunt segregates of low virulence, whereas highly resistant varieties promote the increase of virulent races. Stated differently, one might say that generally susceptible varieties tend to maintain a smut population in equilibrium in conformity with the Hardy-Weinberg law, whereas highly resistant varieties exercise a selection pressure against genotypes, except those compatible with their particular resistance genes. In practical research work, such as the identification of physiologic races, varieties with various types of reaction are assembled to form differential host assortments on which mass collections from the field are often maintained generation after generation on the assumption that selection from varieties of given types of reaction will "purify" a race. Cherewick (1958), however, has found that even after a smut collection had been selected from successive hosts for several generations, and had reached the appearance of constancy on all differential hosts, it was still possible to obtain sporidial combinations virulent to certain hosts that previously had been highly resistant. This type of selection procedure, although it increases the purity of collections, cannot be expected to bring about homozygosity at the many loci governing pathogenicity. To approach that objective it is necessary to use lines selected from individual teliospores.

Mass selection, however, must be employed in many genetic studies. In interpreting the results of such studies it is important to realize that the smaller the population studied, the greater is the role played by chance in selection. If, for instance, an F_2 population should arise from a very few infections, the chances are that most of the genotypes that could arise from the F_1 spores will not find their way into the F_2 generation; an analysis of these results would then give a false impression of the inheritance of pathogenicity in the cross.

B. *The Rusts*

1. *Physiologic Specialization*

The discovery of pathogenic specialization is fundamental to studies on pathogenic variability. Eriksson (1894) showed that stem rust, *Puccinia graminis,* on cereals and grasses was composed of several pathogenically distinct strains which he described as specialized forms *(formae speciales).* Each specialized form was parasitically adapted to particular host plants: f. sp. *tritici* to species of *Triticum* and *Hordeum; f. sp. avenae* to *Avena* species, and so forth. Stakman and his collaborators (Stakman and Piemeisel, 1917a, 1917b; Levine and Stakman, 1918) showed that Eriksson's f. sp. *tritici* could be further subdivided into more narrowly defined pathogenic units (physiologic races) by the use of wheat varieties as differential hosts. That the physiologic races, identified on standard differential host assortments, may be further subdivided by the use of additional hosts has been demonstrated by numerous investigators. Nevertheless, a physiologic race has, on each differential host, pathogenic characteristics that are well defined and relatively constant under conditions optimum for the host-parasite complex and, consequently, its pathogenic characteristics may serve as "characters" of the race in genetic studies.

2. *Sexual Phenomena*

The discovery by Craigie (1927) of the function of the pycnia of the rusts opened the way to hybridization studies. Each of the four sporidia (basidiospores) produced on the promycelium of a germinating teliospore was capable of infection and thereafter of producing pycnia. In each infection the pycniospores present in the exudate of the pycnia are genetical though not morphological, replicas of the sporidium that produced the infection. Since the pycniospores fall into the two groups of mating types, which Craigie described as (+) and (−), it could be inferred that two of the four sporidia on a promycelium were of (+) mating type and two of (−). Since a transfer of pycniospores from (+) pycnia to (−) pycnia or vice versa is necessary to bring about the formation of the dikaryotic aeciospores, it is apparent that this operation is analogous to the mating of two compatible monosporidial mycelia in the smuts.

In long-cycled rusts the nuclear association initiated in the aeciospore persists through the uredial stage and is terminated only by fusion of the associated nuclei in the mature teliospore. As far as is known meiotic behavior in the germinating teliospore is essentially the same as in the teliospore of the smuts.

In experimental work a uredial culture may be propagated indefinitely by successive inoculations of susceptible hosts; and, if it differs pathogenically from other known cultures, it may be properly regarded as a distinct physiologic race whether it is homozygous or heterozygous.

The implication of Craigie's discovery in regard to hybridization studies was obvious. It made possible the "crossing" of physiologic races, that is, the production of new dikaryotic clones, by bringing a haploid nucleus of one race into association with a haploid nucleus of another, and the "selfing" of races, which is merely the reasociation of the products of meiosis of a particular race. Both processes have been applied in several rusts, but actual genetic studies have been carired out chiefly in stem rust, *Puccinia graminis*, and in flax rust, *Melampsora lini.*

3. *Puccinia graminis*

Three different lines of investigation were suggested by Craigie's discovery. (1) Pure cultures of physiologic races could be selfed by intermixing the exudate of pycnia of the same race. (2) The races within a specialized form could be crossed with one another. (3) Crosses could be attempted between a race of one specialized form and a race of another.

a. *The Selfing of Physiologic Races.* Waterhouse (1929) selfed field cultures of wheat stem rust presumed, on circumstantial evidence, to be race 34. The progeny was composed of races 34, 11, and 56. Newton *et al.* (1930a) selfed eight wheat stem rust races and found only one of these to be homozygous for all the pathogenic characters expressed on the twelve differential hosts used for race identification. Of the other seven races all were heterozygous for pathogenicity on one or more of the differential hosts and one was heterozygous also for urediospore color.

Johnson (1954) reported on the selfing of forty-two cultures of wheat stem rust comprising thirty-four physiologic races. Of the forty-two cultures selfed only nine appeared to be homozygous for all pathogenic characters observed. In all of the cultures studied the pathogenic properties expressed on a given differential host appeared to be inherited according to the same principles. On the varieties Kanred and Reliance *(Triticum vulgare)*, Einkorn *(T. monococcum)*, and Vernal *(T. dicoccum)*, avirulence was a dominant character, virulence a recessive one. On the durum wheats Arnautka, Mindum, and Spelmar, virulence was a dominant and avirulence a recessive character. These studies made it clear that the phenotype of a race permits exact prediction of the pathogenicity of the progeny only when the recessive characteristics are expressed on the differential hosts, if dominant characters are expressed, there is no way of predicting whether these are in a heterozygous or homozygous condition.

b. *The Crossing of Races within a Specialized Form.* In crosses between races of wheat stem rust (Johnson and Newton, 1940a) the conclusions about dominance and recessiveness of pathogenic characters were the same as in the selfing studies, i.e., avirulence dominant on Kanred and Vernal, virulence dominant on the durum wheats, Arnautka, Mindum, and Spelmar.

Race analysis in the F_2 and F_3 generations of certain crosses led to the following conclusions. If both parent races were virulent on a given host, the progeny, as a general rule, was also virulent. The parent races generally recurred in segregating generations along with numerous other races which were the result of recombinations of those pathogenic traits by which the parent races differed. A pathogenic trait (infection type, such as necrotic flecks, type 1, 2, or 4 infection) would be suppressed in F_1 only to reappear unchanged in several different races in F_2. Intermediacy, that is, the blending of traits, was relatively uncommon. Apparently the distinctive phenotypic traits, the infection types, must be represented in the rust by genetic factors, and inheritance was chiefly Mendelian, often suggestive of simple inheritance of the characters that separated the parent races. The study of the inheritance of abnormal urediospore color, in certain crosses, showed that spore pigmentation was inherited independently of pathogenicity.

TABLE III

INFECTION TYPES OF *Puccinia graminis* f. sp. *tritici*, RACES 9 AND 36 ON THE WHEATS KANRED, MINDUM, AND VERNAL AND THE RECOMBINATIONS OBTAINED IN THE FIRST AND SECOND GENERATIONS OF A CROSS BETWEEN THE TWO RACES[a]

Generation	Race	Wheat variety		
		Kanred	Mindum	Vernal
Parents	9	0	4	4
	36	4	1	0
First generation (F_1)	17	0	4	0
	1	0	1	0
	9	0	4	4
	11	4	4	0
Second generation (F_2)	15	4	4	4
	17	0	4	0
	36	4	1	0
	52	4	1	4
	57	0	1	4

[a] 0 = No rust produced (host immune).
1 = Minute rust pustules surrounded by small, round, necrotic areas (host resistant).
4 = Large rust pustules without necrotic areas (host susceptible).

The mechanism of inheritance which appears to operate in such crosses can best be gathered from a specific example. The parent races of the cross, race 9 × race 36, differed by contrasting infection types on the host varieties Kanred, Mindum, and Vernal, as shown in Table III, which shows also the infection types of the F_1 hybrid race 17 and of the races isolated in the F_2 population.

Table III shows that infection types of the parent races reappeared in F_2 in the various possible combinations. Because the infection types dominant in F_1 on Kanred and Mindum reappeared in F2 about three times as frequently as those that were recessive, the pathogenicity of the rust on these varieties was considered to be governed by one pair of alleles. Since the dominant infection types on Vernal occurred about 18 times as frequently as the recessive, two pairs of alleles were assumed to be operative. On these assumptions it was possible to explain the distribution of physiologic races in F_2 (Table IV). The F_1 heterozygote, race 17, in which the dominant factors could be present in either the homozygous or heterozygous state, occurred most frequently in F_2, whereas race 52, which expressed all the recessive characters, appeared least frequently, as would be expected. In the heterozygous races a given phenotype might be represented by several genotypes.

Although these studies showed that inheritance of pathogenicity is largely Mendelian, they showed also that it is not exclusively so. Pathogenic characteristics expressed on certain host varieties in F_1 tended to resemble very closely those of the maternal (receptor) parent race used in the cross. This phenomenon was observed in any crosses of a race producing type-2 infection on Marquis with a race producing type-4 infection. When such crosses were made reciprocally, the F_1 hybrids derived from the cross and its reciprocal cross differed strikingly in infection type on Marquis—each showing an infection type resembling that of the maternal parent race (Newton *et al.*, 1930b; Johnson *et al.*, 1934). In crosses between oat stem rust races the same phenomenon was observed on the varieties Joanette and Sevnothree (Johnson and Newton, 1940b). A possible explanation is that the pycniospores of the donor race which bring one nucleus to the new dikaryon do not bring with them the cytoplasm of the paternal parent race. This hypothesis gains some support from the persistence of this phenomenon to a greater or lesser degree in all individuals in the F_2 generation.

c. *Crosses between Specialized Forms.* Stakman *et al.* (1930) showed that it is possible to cross certain of the specialized forms of *Puccinia graminis* and that such crosses may give rise to rusts pathogenically different from the parent rust forms. Two rust strains derived from crosses between f. sp. *secalis* (rye stem rust) and f. sp. *tritici* (wheat stem rust) were less virulent to wheat varieties than the *tritici* parent race and much less virulent to rye varieties than the *secalis* parent race. Johnson *et al.* (1932) found that hybrids between the same two specialized forms were low in virulence on most wheat varieties and on rye but resembled both parent rusts in their ability to attack barley; and Levine *et al.* (1934) reported hybrids even more narrowly limited to barley in their pathogenicity.

Johnson (1949) considered this kind of reduction of virulence a common characteristic of hybrids between specialized forms of stem

TABLE IV

ACTUAL AND THEORETICAL DISTRIBUTION OF PHYSIOLOGIC RACES IN THE COMBINED F_2 GENERATIONS OF TWO CROSSES BETWEEN RACES 9 AND 36, THE THEORETICAL DISTRIBUTION BEING BASED ON THE ASSUMPTION THAT SINGLE-FACTOR PAIRS GOVERN RUST BEHAVIOR OF THE VARIETIES KANRED AND MINDUM AND THAT TWO PAIRS OF FACTORS GOVERN RUST BEHAVIOR ON THE VARIETY VERNAL[a]

Parent Races				F_1				F_2				
								Genotypes	Race	Theor. distribution	Actual distribution	
		Race 9						A B C D A B c D A B C d	17	171.4	165[b]	
Hosts	Krd	Mnd	Ver					a B C D a B c D a B C d	11	57.1	64[c]	
Genotype	AA	BB	ccdd		Krd	Mnd	Ver					
Inf. type	"0"	"4"	"4"	Race 17	Aa	Bb	CcDd	A b C D A b c D A b C d	1	57.1	47	$\chi^2 = 11.37$ P = 0.13
		Race 36			"0"	"4"	"1"	a b C D a b c D a b C d	36	19.0	31	
Hosts	Krd	Mnd	Ver					A B c d	9	11.4	11[d]	
Genotype	aa	bb	CCDD					A b c d	57	3.8	4[e]	
								a B c d	15	3.8	2	
Inf. type	"4"	"1"	"1"					a b c d	52	1.3	1	

[a] From Johnson and Newton, 1940a.

[b] In this group are included six cultures of race 29 which differs from race 17 by producing an "x" infection type on Mindum instead of a "4" type.

[c] In this group are included five cultures of race 32 which differs from race 11 by producing an "x" infection type on Mindum instead of a "4" type.

[d] In this group are included six cultures of race 85 which differs from race 9 by producing an "x" infection type on Vernal instead of a "4" type.

[e] In this group is included one culture of race 110 which differs from race 15 by producing an "x" infection type on Vernal instead of a "4" type.

rust. In crosses between f. sp. *tritici* and f. sp. *avenae*, however, Johnson and Newton (1933) found that the hybrids combined to some degree the pathogenic powers of the parents, resembling oat stem rust in moderate ability to attack oats and wheat stem rust in slight ability to attack certain wheat varieties and barley. Combination in a hybrid of the full pathogenic abilities of both parent forms is probably rare but may occur occasionally, as shown by Levine and Cotter (1931), who described a *secalis* × *tritici* hybrid rust capable of attacking both wheat and rye rather severely as well as barley.

Diminished virulence associated with extension of host range, which occurs in many hybrids between specialized forms, is probably attributable to the fact that the hybrid receives half of its nuclear material from each parent form. The reduced virulence of most hybrids is an obstacle to the establishment in nature of rusts of hybrid origin, but it is not the only obstacle. Johnson (1949) has shown that there is considerable intersterility between some of the specialized forms.

4. *Melampsora lini*

The hybridization work in *Puccinia graminis* showed that the rust organism contained genetic factors which conditioned its pathogenic behavior. In this work the host varieties were considered merely as substrates for the rust races although there was obviously a race-host interaction which differed according to the genes present in the rust races. Since many investigations had shown that host resistance to rust was conditioned by genetic factors, it was logical to assume that there should be a relationship between host genes for resistance and rust genes for virulence or avirulence. Such a relationship, however, could not be shown unless parallel studies were carried out through crosses, on the one hand between resistant and susceptible varieties and on the other hand between virulent and avirulent races. Through his studies of this interrelationship between host factors for resistance and rust factors for virulence, Flor has brought rust genetics to its highest level of scientific interest and practical usefulness.

Basic to Flor's work are two earlier investigations. Henry (1930) showed that immunity to flax rust was dominant to susceptibility and was conditioned by a single pair of alleles in Ottawa 770B and Bombay and by two pairs in Argentine Selection. Myers (1937) reported the presence in flax varieties of two allelic series of genes conditioning rust reaction. The postulation by Myers of two sets of alleles was based principally on the fact that crosses between Ottawa 770B and Newland, both of which were immune from the rust collections with which he worked, produced progeny which segregated immune: susceptible in F_3, thereby necessitating the assumption of two sets of alleles: LL for Ottawa 770B and MM for Newland. In his thirty-seven crosses involving seventeen strains and varieties of flax, he found that immunity was dominant to near-immunity, resistance, and susceptibility, and resistance was dominant to semiresistance and susceptibility.

The nature of the segregation in several of the crosses made it necessary to assume that allelic series existed at the L and M loci. The factors postulated to explain the segregations in the various crosses were LL and MM, duplicate factors conditioning immunity; l^n and m^n conditioning near-immunity, l^n being allelic to L and m^n to M; l^r and m^r conditioning resistance, l^r being allelic to L and l^n, and m^r alleic to M and m^n. In the varieties studied the genotype for Ottawa 770B was $LL\ mm$; Newland $ll\ MM$; C. I. 438 $LL\ m^r\ m^r$; and C. I. 416-3 $ll\ m^n\ m^n$; and C. I. 712 $l^r\ l^r\ mm$.

Flor (1935) selected a group of differential host varieties by means of which he was able to identify fourteen physiologic races. Five years later (Flor, 1940) the number of races had risen to twenty-four. In this latter paper he stated the desirability of investigating "the inheritance of the factors governing different degrees of pathogenicity in the rust organism as well as those governing immunity in the host."

a. *Hybridizing and Selfing of Physiologic Races.* Flor (1942) selfed six physiologic races, of which three were homozygous for pathogenicity expressed on his differential hosts, and he studied the inheritance of pathogenicity in the F_1 generation of crosses between races 9 and 10, 6 and 22, and 22 and 24, and in the F_1 and F_2 generations of crosses between races 6 and 24. In these crosses avirulence was dominant. The F_1 generation, therefore, tended to display the weaker pathogenic traits of the parent races. In F_2 the virulent, recessive characteristics reappeared. On two varieties, Akmolinsk and Bombay, the inheritance of pathogenicity was monogenic, but on the variety Buda it was conditioned by two genes. Varietal crosses had shown that Akmolinsk and Bombay each possessed one gene for host reaction, whereas Buda possessed two such genes. These facts suggested a gene-for-gene relationship between rust genes for virulence and host genes for resistance.

b. *Host-Parasite Interaction.* The methods used by Flor in his subsequent work were designed to make it possible to confirm or disprove the hypothesis of a rust-gene : host-gene relationship; and these methods are, therefore, of importance to an understanding of his work.

These methods involved parallel studies of crosses between hosts that differed in genes governing host reaction and of crosses between rust races that differed in genes governing rust reaction (genes for virulence and avirulence).

In the crosses between the host varieties (Flor, 1947) it was possible by a judicious selection of test races to divide the F_2 population into classes each of which contained plants with the same host genes. In this connection a procedure of fundamental importance was the testing successively of the reaction of the same plant to several races. A plant which was resistant only to the first, third, and fifth race in this sequence obviously was genically different from a plant which was resistant only to the first, second, and fourth race in the sequence. The ratios of resistant to susceptible F_2 plants to a race not attacking the resistant parent provided an indication of the number of host genes operative against that race.

In the crosses between races (Flor, 1946), a race avirulent to a given variety is crossed with a virulent race. The ratio of avirulent : virulent F_2 cultures is, of course, indicative of the genic differences between the two races, but on the assumption of a rust gene for host-gene relationship this ratio will also indicate the number of host genes conditioning resistance to the avirulent race—a 3 : 1 ratio indicating one gene, 15 : 1, two genes, etc.

In these studies crosses between host varieties and crosses between rust races were essential to the acquiring of information on the genetics of host–parasite interaction because, as pointed out by Flor (1956a), "the genes for pathogenicity in the parasite can be identified only by the reaction of specific varieties of the host. Conversely, the genes for rust reaction in the host can be identified only by selective pathogenicity of races of the parasite."

The complementary interaction of host genes and rust genes is shown in Table V, from which the fundamentals of this interaction may be gathered. It will be observed that host-gene : rust-gene interactions produce resistance when both host and rust are represented by dominant genes, but produce susceptibility when the rust is represented by its recessive gene. This is a general rule to which the only exception is the rust gene effective on Williston Brown. On this variety virulence is dominant.

Flor (1947) confirmed the existence of the *L* and *M* allelic series postulated by Myers (1937) and added a third, *N* series. In a study of host genes that condition the rust reaction of 20 flax varieties he was able to identify 21 distinct genes: 8 in the *L*, 7 in the *M*, and 6 in the *N* series. Crossing over, observed only in the *N* series, indicated that the *N* genes were distributed between two groups of linked alleles on the chromosome bearing the *N* genes. Flor (1955) retained the symbol *N* for those genes allelic to the rust conditioning gene in Bombay and assigned the symbol *P* to genes at the second locus. Apart from these four series there is evidence for an independently inherited gene *K*, present in the variety Clay. Since the genes in the rust organism can be detected only by means of genes present in the host, it follows that the known rust and host genes will correspond in number, the total given by Flor (1956a) being 25.

TABLE V

COMPLEMENTARY INTERACTION OF HOST GENES AND RUST GENES[a]

Segregation of F_2 Plants of Ottawa 770B × Bombay for reaction to Races 22 and 24 of Flax Rust

Race and pathogenic genotype	Reaction* and genotype of					
	Parent varieties		F_2 plants			
	Ottawa 770B *LLnn*	Bombay *llNN*	*LN*	*Lnn*	*llN*	*llnn*
Race 22, $a_La_LA_NA_N$	S	I	I	S	I	S
Race 24, $A_LA_La_Na_N$	I	S	I	I	S	S
Number of plants observed			110	32	43	9
Number of plants expected (9:3:3:1)			109	36	36	12

$\chi^2 = 2.563$; $P = 0.30$ to 0.50

*I = immune; S = susceptible

Segregation of F_2 Cultures of Race 22 × Race 24 of Flax Rust for Reaction to Ottawa 770B and Bombay

Variety and reaction genotype	Reaction* of variety to					
	Parent race		F_2 genotype			
	22 $a_La_LA_NA_N$	24 $A_LA_La_Na_N$	A_LA_N	a_LA_N	A_La_N	a_La_N
Ottawa 770B, *LLnn*	S	I	I	S	I	S
Bombay, *llNN*	I	S	I	I	S	S
Number of cultures observed			78	27	23	5
Number of cultures expected (9:3:3:1)			75	25	25	8

$\chi^2 = 1.565$; $P = 0.50$ to 0.70.

*I = immune (avirulent); S = susceptible (virulent)

[a] From Flor, 1956a.

The discovery of further host and rust genes may be expected especially from studies in other countries in which race composition differs from that in North America. Kerr (1952) has located host genes such as

*L*9 which cannot be identified by means of currently known North American races. Generally, his studies have confirmed the allelic series established by Myers and Flor. A corollary of the complementary gene hypothesis is that the number of loci at which host genes occur determines the number of resistance genes that can be incorporated in one flax variety. Since there are only five such loci, the number of resistance genes that can be built into one variety, on the basis of present knowledge, is five. In the rust no corresponding series of alleles is known, although evidence exists that some of the genes are closely linked (Flor, 1946). There appear, therefore, to be no limits to the number of genes for virulence that may occur in one race of the rust.

TABLE VI

UTILIZING RACES TO IDENTIFY THE RUST-CONDITIONING GENES IN THE PROGENY OF HYBRIDS OF OTTAWA 770B, CASS, AND POLK[a]

Race number	Reaction[b] of plants possessing genes							
	llmmnn	L	M^3	N^1	LM^3	LN^1	M^3N^1	LM^3N^1
156 a_La_L A_{M^3} $a_{N^1}a_{N^1}$	S	S	R	S	R	S	R	R
192 A_L $a_{M^3}a_{M^3}$ $a_{N^1}a_{N^1}$	S	R	S	S	R	R	S	R
154 a_La_L $a_{M^3}a_{M^3}$ A_{N^1}	S	S	S	R	S	R	R	R

[a] From Flor, 1955.

[b] R = Resistant; S = susceptible.

Flor's method, already briefly mentioned, of selecting from the progeny of varietal crosses those plants that contained single rust conditioning genes, has important consequences for the development of resistant varieties and for the identification of rust races. By these methods, described in detail by Flor (1955), it is possible to separate these genes in such a way that one flax variety contains a single rust conditioning gene. Since these genes in the host are specific for genes in the rust, it becomes possible to develop differential host assortments permitting a highly accurate pathogenic analysis of the rust. Conversely, it becomes possible, once the host genes have been separated into different flax varieties, to combine these genes in the various possible ways in new flax varieties to provide the maximum possible resistance against rust races. The effectiveness of this process, as well as the interaction of rust genes with host genes, can be seen from Table VI.

A consideration of the protective effect of several genes combined in one variety shows the essence of the hypothesis to be that the variety remains resistant unless a given rust race contains pathogenicity factors that can overcome the protective effect of *all* of the host genes. Several separate races, each containing one of the necessary pathogenicity factors, would not overcome the resistance of the host plant because each of these races possesses only the physiological processes necessary to interlock with one of the resistance mechanisms of the host. The remaining mechanisms give protection so long as a race does not possess all of the interlocking mechanisms.

The work of Flor has been criticized by Mayo (1956) chiefly on the basis that the foundations on which it rests, the allelic series established by Myers and Flor, and the one-for-one relationship between rust and host genes, are not soundly established, and he has suggested "more critical tests for allelism and linkage between both host genes and pathogen genes."

C. *The Ascomycetes*

1. *The Ascomycetes as Subjects for Genetic Studies*

In the Basidiomycetes the principal pathogenic stage is dikaryotic (functionally diploid); in the Ascomycetes it is haploid. The dikaryotic phase, the major portion of the life cycle in the Basidiomycetes, is, in the Ascomycetes, a transient phase subsequent to fusion of the mycelia of two haploid thalli (in heterothallic species) or following sexual differentiation in the same thallus (in homothallic species). In both types of fungi the true diploid phase is limited to a brief premeiotic period confined in the rusts and smuts to the mature teliospore and in the Ascomycetes to the young ascus. Following meiosis and the production of ascopores, the pathogenic phase commences with the germination of the ascospore and ends with the formation of perithecial initials.

Since the pathogenic phase of an ascomycete is haploid and, therefore, contains only one of each pair of homologous chromosomes, any mutation that occurs is immediately expressed unless its expression is suppressed by genic interaction, such as epistasis, as in some of the nonpathogenic mutants in *Venturia inaequalis* reported by Keitt *et al.* (1943).

By reason of their haploidy, ascomycetous fungi are organisms suitable for the production of induced mutations as has been shown for *Neurospora* by Beadle and his co-workers and for *Venturia* by Keitt and his collaborators. This is a matter of much significance, as the study of biochemical mutations opens up a promising approach to a better understanding of the mechanism of genic control of pathogenicity.

Not least important in establishing the popularity of the Ascomycetes as tools for genetic studies is the fact that all the products of meiosis are present in the ascus and may be segregated for study, in some species in the linear order in which they are laid down in the ascus. The frequency of first- and second-division segregation may thus be determined and calculations made of the distances of various loci from their centromeres.

2. *Inheritance of Pathogenicity in Venturia inaequalis*

Outstanding among the investigations on the inheritance of pathogenicity in the Ascomycetes are the studies of Keitt and his collaborators on the apple scab fungus *Venturia inaequalis*. Since the historical development of these studies has been reviewed adequately by Keitt (1952) and Keitt and Boone (1954), mention will be made here only of the principal studies relating to the inheritance of pathogenic characters.

Keitt (1952) states that it was observed as early as 1926 that some cultures of the fungus produced typical scab lesions on apple leaves, whereas other cultures produced only flecks. Wiesmann (1931) and Palmiter (1932) showed that monoconidial isolates differed in their pathogenic capabilities, and Palmiter (1934) concluded that monoconidial isolates generally differed from one another in cultural characteristics and in infection capabilities. Keitt and Palmiter (1938) showed that the fungus was heterothallic, the eight ascospores of an ascus falling into two groups of opposite mating types. It was now apparent that the existence of contrasting pathogenic characters in a heterothallic fungus provided inviting opportunities for genetic studies on pathogenicity.

Keitt and Langford (1941) developed a technique for isolating in serial order the eight ascopores of an ascus. They showed that the single-spore isolates were hermaphroditic but self-sterile and that they belonged to one or the other of two mating-type groups in which intragroup matings were sterile and intergroup matings fertile. They also developed methods for growing and mating cultures *in vitro* and for testing the pathogenicity of cultures under controlled greenhouse conditions on a group of differential varieties. In this study it was shown (for the first time for an ascomycete) that segregation for pathogenicity frequently occurred in the eight monosporous cultures of a single ascus, four cultures producing lesions and four producing flecks on a differential variety. They concluded that this 1 : 1 segregation for pathogenicity, and for other characters, occurred sometimes in the first and sometimes in the second meiotic division, whereas the third division was equational. Encouraging for future genetic studies was the pathogenic stability of lines eliciting the lesion and fleck reactions.

The inheritance of the sharply contrasted pathogenic characters, lesion and fleck, was studied by Keitt *et al.* (1943). They found that when two normal, wild type lines that produced lesions on a given variety were crossed, all the resulting ascospore cultures produced the lesion reaction. Crosses of fleck × fleck produced only the fleck reaction.

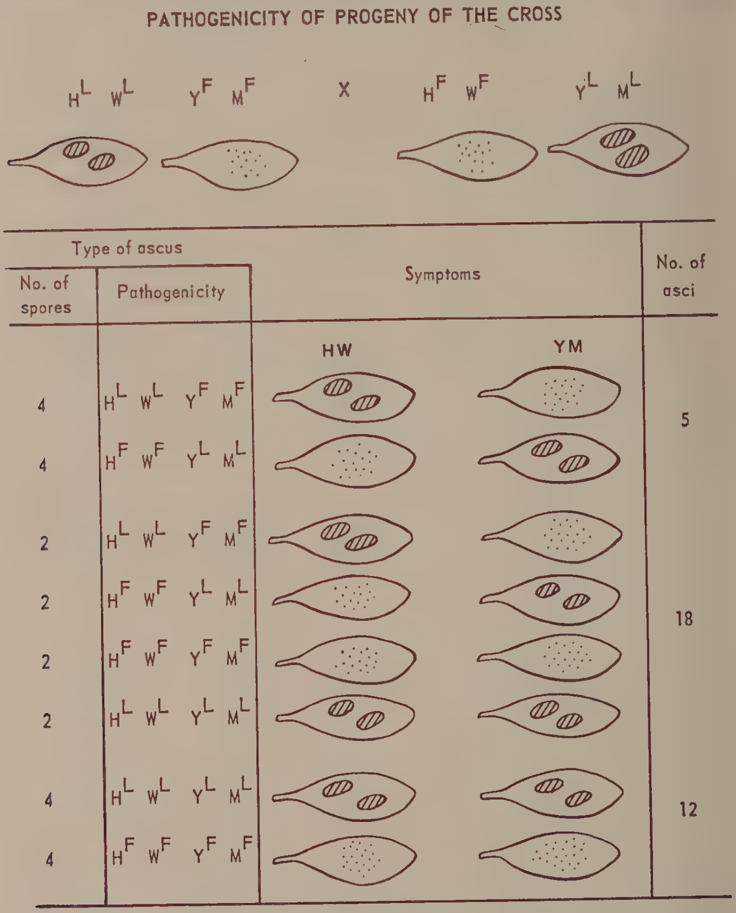
PATHOGENICITY OF PROGENY OF THE CROSS
H^L W^L Y^F M^F
X
H^F W^F Y^L M^L
Type of ascus
No. of spores
Pathogenicity
Symptoms
No. of asci
HW
YM
4 H^L W^L Y^F M^F
4 H^F W^F Y^L M^L
5
2 H^L W^L Y^F M^F
2 H^F W^F Y^L M^L
2 H^F W^F Y^F M^F
2 H^L W^L Y^L M^L
18
4 H^L W^L Y^L M^L
4 H^F W^F Y^F M^F
12

FIG. 3. Inheritance of pathogenicity in the progeny of thirty-five asci derived from crosses between pathogenically distinct lines of *Venturia inaequalis*. (Adapted from Keitt *et al.*, 1948.)

Crosses of lesion × fleck produced a 1:1 ratio of cultures with lesion and fleck symptoms.

Figure 3, adapted from Keitt *et al.* (1948), shows the inheritance of the lesion and fleck reactions in a cross of a line producing lesions on the varieties Haralson and Wealthy but flecks on Yellow Transparent and McIntosh with two lines producing the converse reactions. The progeny of the thirty-five asci studied showed only the lesion and fleck reactions, occurring in a 1 : 1 ratio. Both parental types occurred in the progeny as well as the two expected recombinant types. Analysis led to the conclusion that pathogenicity to Haralson and Wealthy was determined at one locus and to Yellow Transparent and McIntosh at another.

Shay and Williams (1956) proved the existence of three physiologic races and demonstrated that the pathogenicity of one of these was controlled by three different genes in the fungus.

Boone and Keitt (1957) investigated ten pathogenically different lines and showed that pathogenicity on the apple varieties tested was governed by seven or more gene pairs in the fungus, each pair consisting of an allele conditioning the lesion reaction and another conditioning the fleck reaction. At least four of these gene pairs occurred at separate loci. Alleles conditioning flecks were found to be epistatic to those conditioning lesions.

Williams and Shay (1957) assigned the symbols $p\text{-}1^{+}/p\text{-}1$ * through $p\text{-}7^{+}/p\text{-}7$ to the above-mentioned gene pairs and added five more gene pairs, $p\text{-}8^{+}/p\text{-}8$ to $p\text{-}13^{+}/p\text{-}13$, effective against the apple varieties and species they tested. Studies on host genes conditioning resistance indicated the probability of single-gene control.

In the studies by Keitt and Langford (1941) many cultural mutants occurred in certain of the monoascospore isolates, usually as colony sectors. These mutant lines were relatively stable and were invariably less pathogenic than the lines from which they originated. Some of the mutant characters affecting pathogenicity were studied by Keitt *et al.* (1943) by analyses of the progenies of crosses of *normal* × *tan* and *normal* × *tan nonconidial.*

The mutant *tan* (T) differed from *normal* (t) by the tan color of its colonies and by reduced conidial production. Infection tests showed that it suppressed all visible expression of pathogenicity.

The mutant *nonconidial* (Nc) produced no conidia and suppressed visible expression of pathogenicity except on the variety McIntosh, on which the lesion reaction was modified to fleck.

The cross *normal* × *tan* produced four lines normal and four lines tan from each ascus, the lines carrying tan being noninfectious. The fact that two of the four normal lines had the pathogenicity of the original normal line from which the *tan* mutant arose indicated that the mutation to *tan* did not change the factor for pathogenicity but merely suppressed its expression.

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* + Indicates full virulence to a given variety; the other allele conditions a lower virulence.

The results of the cross *normal* (t nc) × *tan nonconidial* (T Nc) are shown in Table VII. The parental types occurred in the progeny as well as the recombinants *tan* (T nc) and *nonconidial* (t Nc), which segregated from their alleles in a 1 : 1 ratio. All lines carrying *tan* were nonpathogenic on all varieties, and lines carrying nonconidial without *tan* were nonpathogenic except to McIntosh on which they produced the fleck reaction. The fact that the two normal cultures in the progeny had the same pathogenicity as the original normal culture from which the mutant *nonconidial* was derived showed that this mutation did not arise at the locus for pathogenicity.

TABLE VII

INHERITANCE OF PATHOGENICITY AND CULTURAL CHARACTERISTICS IN THE CROSS NORMAL (t nc) × TAN NONCONIDIAL (T Nc), ASCUS N[a]

Pathogenicity genotypes: Normal—MC H MP yt ra × MC H MP YT RA[b]

Ascospore no. and cultural type	Host variety and pathogenicity					Genotypes					
	MC[b]	H	MP	YT	RA	Pathogenicity					Cultural characters
1 Tan	O	O	O	O	O	MC	H	MP	?	?	T nc
2 Tan	O	O	O	O	O						
3 Tan Nonc	O	O	O	O	O	MC	H	MP	?	?	T Nc
4 Tan Nonc	O	O	O	O	O						
5 Normal	L	L	L	L	L	MC	H	MP	YT	RA	t nc
6 Nonc	F	O	O	O	O	MC	H	MP	?	?	t Nc
7 Normal	L	L	L	L	L	MC	H	MP	YT	RA	t nc
8 Nonc	F	O	O	O	O	MC	H	MP	?	?	t Nc

[a] Adapted from Keitt *et al.*, 1943.

[b] MC-McIntosh, H-Haralson, MP-Missouri Pippin, YT-Yellow Transparent, RA-Red Astrachan.

As may be seen from Table VII the presence of the mutant genes brought about pathogenicity ratios of 2 : 1 : 1 (for McIntosh) and 3 : 1 (for the other varieties). The mutant genes, therefore, acted as modifiers of pathogenicity ratios.

A gene in a pathogenic organism sets in motion biochemical processes, the end result of which is a particular pathogenic reaction. This realization stimulates the investigator to seek an understanding of the nature of these processes if any means to that end appear to be available. One such means, originally developed by Beadle and Tatum for the fungus *Neurospora*, is the method of inducing mutations and studying their effect on biochemical processes. In recent studies, Keitt and his co-workers have applied these methods to *Venturia* in an effort to elucidate the gene-controlled chemical processes associated with pathogenicity.

Mutations were induced in a monoascosporic wild-type line with either nitrogen mustard or ultraviolet irradiation (Keitt and Boone, 1954). The induced mutants were of three general types: morphological, color, and biochemical. Morphological mutants, the most numerous, were of least use for the physiological studies. The color mutants, useful for chromosome mapping (Boone and Keitt, 1956), fell into three linkage groups. Ten of the twelve mutant characters studied appeared to be governed by a single gene each. The biochemical mutants, of greatest interest in relation to pathogenicity, were subjected to determination of the nature of their growth deficiencies. The mutants fell into four classes according to the chemical substances for which they were deficient: *vitamins*—nicotinic acid, biotin, inositol, pantothenic acid, choline, riboflavin; *nitrogen bases*—pyrimidines, purines; *amino acids*—arginine, lysine, histidine, methionine, proline; *reduced sulfur.*

Genetic studies with fifty-four of the biochemical mutants showed that in crosses with wild type the mutant characters segregated in a 1 : 1 ratio, indicating a single-gene control. The synergism method similar to that applied by Beadle and Coonradt (1944) to *Neurospora* was used to establish whether two or more mutants for the same deficiency were determined at the same locus or at different loci, and data from percentage of second-division segregation were used to determine the distances of loci from their centromeres. It was shown (Boone *et al.*, 1957) that mutations for the same deficiency were frequently controlled at different loci. The number of genes concerned with each of the following nutritional requirements were: riboflavin, 3; purines, 8; pyrimidines, 4; arginine, 6; histidine, 2; reduced sulfur, 2; all others, 1.

In relating these results to pathogenicity it was necessary to determine whether or not a given nutritional deficiency influenced the pathogenicity of the mutant. If a given deficiency inhibited pathogenicity, it was necessary to determine whether pathogenicity could be restored by supplying the particular substance which the fungus could not synthesize to the host plant during or after infection. In this connection the amount of the substance present in the host was also a matter of possible significance.

The testing of the pathogenicity of the induced biochemical mutants showed that mutants requiring, respectively: biotin, inositol, nicotinic acid, pantothenic acid, or reduced sulfur were pathogenic; mutants requiring choline, riboflavin, purines, pyrimidines, arginine, histidine, methionine, or proline were nonpathogenic. The reasons for supposing that the losses of pathogenicity were due to the deficiencies were (1) the mutants were auxotrophic; (2) mutants with like deficiencies showed like losses of pathogenicity; (3) the mutant progeny from crosses of nonpathogenic mutants with wild type were nonpathogenic, whereas the wild type progeny from the same crosses were pathogenic.

The test of whether or not pathogenicity could be restored to a mutant by supplying the deficient metabolite (Kline *et al.*, 1957) to the host plant during various periods from inoculation onward showed that pathogenicity was temporarily restored, wholly or in part, to six of the eight mutants tested if application of the required nutrients was made daily during the incubation period. The mutants that recovered pathogenicity by these means were those deficient, respectively, for choline, riboflavin, pyrimidines, arginine, histidine, or methionine. Histological studies showed that although a mutant could, without supplement of nutrient, penetrate the cuticle, it could make little further growth in the plant tissues unless it were supplied with the particular metabolite for which it was deficient. The two mutants to which pathogenicity was not restored by nutrient supplement were one requiring adenine or hypoxanthine and one requiring either guanine or xanthine. Studies of the chemical composition of the host tissues showed that the chemical substances for which the mutants were deficient were present in the host, but at the site of infection they occurred in quantities below the requirements of the fungus.

IV. Transformation and Transduction

Recombination resulting from some sort of sexual process, shown by Lederberg and his collaborators to function in certain strains of *Escherichia coli*, is not known to be operative in plant-pathogenic bacteria. Transformation, now known to function in phytopathogenic bacteria, however, may play much the same role as sexuality in the transfer of genetic materials from one strain of bacteria to another. In "transformation" a bacterial clone acquires some of the characteristics of another clone by growing in its extract or filtrate. "Transduction," originally referred to as phage-induced transformation, (Zinder and Lederberg, 1952) was defined by Demerec *et al.* (1954) as: "a process whereby a phage particle transfers a segment of chromosome from the bacterium in which it was raised to the bacterium which it infects. After infection, this segment is in some way incorporated into the corresponding chromosome of the recipient bacterium, replacing a homologous region, and is so transmitted to the descendants of that bacterium." Some recent authors (cf. Cavalli-Sforza, 1957) refer to transformation as "naked, or DNA-transduction" and designate the phage-induced transformation as "phage-mediated transduction." The two processes are not fundamentally different: in both the transforming principle is deoxyribonucleic acid (DNA); in both the end result is the introduction into a strain of bacteria of one or more characters of another strain.

Transformation, first recorded by Griffith (1928) for pneumococcus and studied intensively by Avery and his collaborators, was shown by Hotchkiss (1951) to result in the independent transfer of several distinct characters from one pneumococcus strain to another. Corey and Starr (1957a, b) reported that they had effected genetic transformation of colony type in *Xanthomonas phaseoli*. In this species four types of colonies occur: rough, smooth, semimucoid, and mucoid. Although these different types multiply equally well within the host, they differ in colony size on nutrient media and in their ability to produce lesions. The amount of polysaccharide in the bacteria increases according to the above sequence, being smallest in the rough and greatest in the mucoid types, and the size of lesions follows the same sequence.

In the interconversion of the types the amount of polysaccharide, and the degree of pathogenicity, appear to be dependent on three genes: A, B, and C. The nonfunctional alleles a, b, and c would be present in the rough colonies; smooth, semimucoid, and mucoid cells would have progressively increasing numbers of A, B, and C genes (smooth Abc, aBc, and abC; semimucoid ABC, AbC, aBC; mucoid ABC). Transformation by DNA from rough cells is in the direction of decreased polysaccharide production, and transformation from mucoid cells is in the opposite direction.

Somewhat similar interconversions have been effected between *Agrobacterium (Phytomonas) tumefaciens* and related bacteria. Coleman and Reid (1949) brought about a conversion *in vitro* of *A. tumefaciens* to the morphologically similar but nonpathogenic *Agrobacterium (Alcaligenes) radiobacter* by placing the capsule of the latter on the noncapsulated phase of *A. tumefaciens*. Conversely, "the removal of the *A. radiobacter* "M" capsule changed the organism from a state of avirulence to one of significant virulence."

Klein and Klein (1953) transmitted tumor-inducing ability from virulent crown gall bacteria to avirulent strains of *A. tumefaciens*, and to *A. rubi, A. radiobacter*, and *Rhizobium leguminosarum*. Once acquired by the recipient organism, virulence appeared to be genetically fixed. Klein and Klein (1956) found that filtrates of virulent, donor cultures contained deoxyribonucleic acid, which acted as the transforming principle in conferring virulence on recipient cultures. The tumor-inducing principle was considered to be a metabolic product of virulent crown gall bacteria, its production being governed by one or more genetic factors which can be transmitted to or evoked in deficient forms.

V. Discussion

This survey of the inheritance of pathogenicity has shown that the disease-producing capacities of pathogenic fungi with a functioning sexual process are quite commonly gene controlled. In these fungi recombination undoubtedly helps to confer a pathogenic plasticity on the organism. In organisms without a functioning sexual process, including many fungi and probably all plant-pathogenic bacteria and viruses, mutation appears to provide the chief means of pathogenic adaptability to the host. In these organisms the absence of sexually determined recombination is probably only to a slight extent counterbalanced by processes such as heterokaryosis and parasexuality. Although the last-mentioned of these processes, and others such as transduction, have not yet been proved to be operative in many plant-pathogenic organisms, they can not be entirely ignored as means of pathogenic variation. But even if these processes are not commonly operative, mutation and selection appear to be adequate means for pathogenic variation in most non-sexual organisms. There appears to be little, if any, proof of the nature of mutation for pathogenicity. Presumably, many of these changes are gene mutations, but as stated in the Introduction, the term mutation is here used for any heritable change that is not demonstrably due to other means, such as recombination, heterokaryosis, transduction, or parasexual processes.

In this review several examples have been given of a close interrelationship between pathogenic strains, often of mutant origin, and host genes conditioning the reaction of the host. It is perhaps advisable to point out that these relationships can, in most cases, only be claimed to be host-gene : pathogen-strain relationships because there are few demonstrations that the pathogenicity of strains is conditioned by a single gene. Exception, however, can be made of the work of Flor, who has claimed for flax and flax rust a rust-gene : host-gene interaction. It is tempting to assume such a relationship also for instances in which a strain ineffective against a host gene mutates to a strain effective against it. Such mutations, as in *Cladosporium fulvum* and *Phytophthora infestans*, are quite likely to be gene mutations, in which case the new interaction might be considered to be a host-gene : pathogen-gene interaction—but there is no proof of the exact nature of the changes that took place in the mutations and, consequently, there is no proof of the exact nature of the relationship. Even if the host-pathogen interaction is not a simple gene-for-gene interaction, it seems warrantable to assume that genically controlled processes of the pathogen are interrelating with genically controlled processes in the host. To this extent the widespread host-gene : pathogen-race interaction may be interpreted to support Flor's contention.

One beneficial result of studies on host-pathogen relations is that an understanding of them sometimes enables the investigator to manipulate the resistance genes of the host by means of hybridization in such a way as to provide maximum control of the races. How effectively this can be done has been shown by Flor (1955).

Of greater scientific interest is the possibility that genetic studies may give some vantage point for a better understanding of physiological processes. Genetic studies, by themselves, can not provide knowledge of this kind. Applied to pathogenicity, which is a two-system (host and pathogen) physiological process, genetic studies with both components can provide, and have indeed already provided, knowledge of a host-pathogen interaction that requires thinking in terms of physiology. It is certain that without such thinking the working hypotheses necessary for future physiological investigation will not be forthcoming.

The survey of host-pathogen relations given in this chapter gives reason to suppose that the interrelations of gene-controlled physiological processes of host and pathogen are a basic consideration in obligate parasitism. Physiological studies, in the past, have been concerned chiefly with phenomena common to parasitism, such as the effect of the pathogen on assimilation, respiration, levels of soluble and insoluble nitrogen, etc. The demonstration of the interaction of gene-controlled processes of host and pathogen emphasizes the need of investigating the physiology of the specific phenomena underlying specialized parasitism.

Actually, the demonstration that there are gene-initiated host-pathogen interactions gives no indication as to their nature. Catcheside (1951) points out that host resistance and pathogen avirulence are generally dominant characters. Assuming that dominance implies the production of a substance and recessiveness its absence, he supposes that the necrotic, resistant-type reaction results from the interaction of these two dominant-gene-conditioned substances, related to one another in the manner of antigen and antibody. Since recessive genes in host and pathogen would produce no substance, there would be no such interaction, and hence compatibility, if one or both of the interacting genes were recessive. A similar suggestion is put forward by Flor (1956a).

A different explanation could be made on the assumption that a certain substance is necessary for successful pathogenic relations with the host. If this substance is absent from the host, the parasite would be pathogenic only if it is capable of synthesizing the substance. Those strains incapable of this synthesis would be avirulent, except on those hosts containing the required substance. No assumption need be made of the relation of dominance to substance production.

In considering pathogenicity in terms of gene action, thinking will have to be based on the results of studies with biochemical mutants. Anabolic and catabolic reactions proceed along metabolic paths under the guidance of gene-controlled enzymes. Recent research (Davis, 1955) appears to uphold the original hypothesis of Beadle and Tatum that an auxotrophic mutant lacks a specific enzyme. A metabolic path may require for its completion many enzymes, each catalyzing a step from one intermediate substance to another and each presumably controlled by a gene. A mutation at any of these steps would prevent the completion of the path. Perhaps this is the reason for the finding by Boone *et al.* (1957) that genes at several different loci control the same deficiency. Since these deficiencies limited the pathogenicity of the mutant strains of *Venturia inaequalis*, it is tempting to try to explain the different pathogenic potentialities of parasitic strains in terms of mutations affecting metabolic paths.

If pathogenic differences between strains are based on different nutritional requirements, mutations for decreased virulence might be of the auxotrophic type leading to a difficulty on the part of the pathogen to control a metabolic path. Mutations to increased virulence might be explained by the pathogen's gaining or regaining control over a metabolic path. On these assumptions the relationship of the pathogen to the host would be nutritional. The genes governing host resistance would be resistance genes because they fail to condition metabolic paths, creating the type or quantity of some metabolite required by the pathogen. But because the metabolic paths of both pathogen and host are gene controlled, there would appear to be a gene-for-gene relation when both host and pathogen are studied genetically.

Even if such a direct nutritional relationship should operate, it would be likely to be upset by factors that are not directly nutritional. This has been realized by those who have theorized about host-pathogen relationships. Garber's "nutrition inhibition hypothesis" (Garber, 1956) and Lewis' "balance hypothesis of parasitism" (Lewis, 1953) both consider nutrition to be basic to successful parasitism but liable to be affected by the presence of host metabolites unfavorable to the growth of the pathogen. An example is given by Garber and Shaeffer (1957) who considered that failure of two of their histidine-requiring mutants of *Erwinia aroideae* to grow in the presence of adequate histidine concentration in the host was due to the presence of certain amino acids antagonistic to growth. The inhibition of onion smudge development in red-skinned varieties of onion (Walker and Stahmann, 1955) is another example of the interference with nutrition by nonnutrient chemicals present in the host.

Gene interaction among pathogen genes undoubtedly brings about as yet unexplained reactions inhibitory to pathogenic development. A good example of genically controlled inhibition of pathogenicity is the effect of the mutant gene *tan* on pathogenicity of *Venturia inaequalis*. Evidence was obtained by genetic studies that this inhibition was caused by mutation at a locus other than that of pathogenicity. It seems likely that epistatic effects on pathogenicity are frequently of the chemically inhibitory type.

Gene interaction may, however, have the opposite effect—that is, lead to a synergistic effect evidently necessary in some cases to successful parasitism.

Much of the pathogenic variability displayed by races of a single species is due to the different genic content of the races. Johnson (1953) suggested that it would be simplest to account for the physiologic specialization of the rusts by assuming that rust races have different gene-controlled nutrient requirements. The nutrient substrates that could be acted on by a given race would depend on the gene-controlled enzymatic systems of the race. Some races would have many such systems; others would have few. Races with many enzyme systems would have a versatility with respect to food substrates that would allow them to parasitize many genically different hosts. In such races the full complement of enzymes might not be operating against any one host. A race might operate system A on one host, system B on another, etc.

Hypotheses are chiefly useful as vantage points for experimental work. Nutritional hypotheses of the type outlined above at least have the merit of being susceptible to the testing of their validity, especially in those pathogenic microorganisms that can be grown on artificial media. Recent studies on the relationship of biochemical mutations to pathogenicity in *Venturia inaequalis* have already shown that such working hypotheses may lead to important physiological studies.

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CHAPTER 12

Virus Inactivation *in vitro* and *in vivo*

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I. Introduction

Many agents cause viruses to lose their characteristic biological and physical properties and can interfere with their establishment and multiplication in the plant. The study of the effects of such agents has played an important part in the development of our understanding of viruses ever since they were first discovered about 70 years ago. The resistance of a virus to aging at room temperatures and to heating for short periods in expressed plant sap were important criteria for the identification and classification of viruses before more modern methods began to become available about 25 years ago. Even now, such criteria prove useful for many viruses which have not been isolated and characterized by more precise methods.

Nearly all our knowledge about the inactivation of viruses has been obtained from experiments with those which can be fairly readily transmitted by mechanical means. It is probable that such viruses are generally more stable than those which cannot be mechanically transmitted. Since most of the three hundred odd plant viruses so far recognized fall into this latter group, the material presented in this chapter probably gives a distorted picture of the stability of plant viruses in general.

The knowledge that the infectivity of many viruses in expressed sap could be destroyed by the kind of chemical and physical agencies which were already known to destroy enzymes suggested that viruses might also be proteins. This knowledge led to the first isolation and characterization of tobacco mosaic virus and potato virus X as proteins.

The most outstanding discovery from recent work on plant viruses is that ribonucleic acid (RNA) isolated from tobacco mosaic virus (TMV), and free from detectable amounts of protein, is capable by itself of initiating new infections and of giving rise to new virus complete with the normal coat of protein. It was known in 1936 that several plant viruses were nucleoproteins, but it took another 20 years before the vital role of the nucleic acid was demonstrated directly. For the greater part of this period most investigators were preoccupied with the protein part of the virus. Thus, in studies on virus inactivation the virus protein has been given most attention. Only in fairly recent work has the RNA been given adequate consideration.

This preoccupation with the virus protein persisted in spite of the fact that a number of early experiments showed that the protein part of TMV could be fairly substantially altered without affecting biological activity. For example, 70% of the amino groups of TMV could be covered with acetyl or phenylureido groups without significantly altering the infectivity of the virus (Miller and Stanley, 1941). In the period between 1937 and 1940 treatment with detergent and with phenol was shown to split the nucleic acid from the protein part of several viruses. It is an interesting comment on the influence of public opinion on the development of a subject, that almost 20 years passed before infectious RNA was prepared from TMV by methods using detergent and phenol.

The term "inactivation" has been used with various meanings by different writers. Here we shall use the word in a rather loose way to refer to any treatment which causes the virus to lose its ability to infect or to multiply in the plant, regardless of whether the loss is reversible or whether other characteristic properties of the virus particle are lost.

A wide variety of chemical and physical treatments will inactivate viruses *in vitro*. Many of these—for example strong acids and bases—inactivate rapidly with a total loss of all characteristic properties of the virus. Such treatments are normally of no great interest, and as far as inactivation *in vitro* is concerned we will deal mainly with agents which, under some conditions at least, cause a loss of the ability to infect without total destruction of virus structure.

If a virus preparation is given some treatment and the infectivity decreases with increasing time of treatment, it is usually safe to assume that inactivation of the virus is taking place *in vitro*. However, some substances which inhibit virus infection have their full effect immediately on being mixed with the virus and the inhibition can be annulled by removal or dilution of the inhibitor. With these it is often impossible to distinguish with any certainty between *in vitro* and *in vivo* effects. Some agents such as heat can inactivate viruses both *in vitro* and within the plant. From such considerations it has been found most convenient in arranging the material in this chapter to consider together all the effects of a given inactivating agent.

Many plant species are resistant to infection with a particular virus, and the resistance of susceptible species can be altered by environmental factors such as light, temperature, and nutrition. The nature of resistance is not understood, but in many situations it probably involves inactivation of the virus *in vivo*.

When considering the host plant, the concept of changing resistance or susceptibility to a virus is a useful one. However, from the point of view of the virus the idea seems to have led to some confusion. In the literature on virus inactivation there are many discussions concerned with the problem of whether a particular treatment reduces the number of local lesions produced by a standard inoculum through effects on the virus or on the host. We suggest that this may be a false distinction, and that in most situations, a decrease in the number of local lesions produced by a standard inoculum should from the point of view of the virus be considered as virus inactivation.

When inoculated onto the plant, those virus particles which find suitable points of entry rapidly undergo some irreversible change which prepares them for initiating multiplication of the virus. This change very probably involves the removal of the protein coat from the nucleic acid. Any situation in which the virus is deceived, so to speak, into taking the first irreversible step toward multiplication under conditions where the process cannot continue to the production of new stable particles should be considered as virus inactivation. It is probable that the only kinds of change in the host leading to increased resistance which do not involve virus inactivation are those which give the leaf surface increased resistance to wounding. Here the virus particle which would have invaded remains presumably unchanged in a stable state on the leaf surface and, in theory at least, lives to fight another day.

II. TEMPERATURE

The structure of a virus particle may be altered by the effects of thermal agitation. If such an alteration destroys the ability of some vital part of the structure to function, the virus particle becomes inactivated. In an infected plant, under normal growing conditions, we assume that most of the virus particles can resist thermal inactivation. If, however, plants are grown at a higher temperature than normal, or the virus is extracted from the plant and subjected to higher temperatures, inactivation may occur.

A. *Heating in vitro*

From the early years of plant virus research, the "thermal death point" has been used as a property for virus identification. Usually small volumes of fresh sap from an infected plant are heated in small tubes for 10 minutes, and inoculated into suitable host plants. The thermal death point ranges from about 42° * for tomato spotted wilt virus to about 93° for TMV. Concentration of virus, pH and composition of the sap, and condition of the test plants may affect the results of such tests.

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* All temperatures given as degrees centigrade.

For more careful study of heat inactivation purified virus preparations have been used where possible.

With some viruses such as TMV and potato virus X heat inactivation is closely associated with denaturation of the virus protein. The temperature coefficient of inactivation or Q_{10} (i.e., the ratio between the rate of inactivation at two temperatures, 10° apart) is large. This is a characteristic of protein denaturation in general. With tomato bushy stunt and tobacco necrosis viruses, however, the Q_{10} for heat inactivation is small, and there is a fairly wide range of temperatures at which some infectivity is lost. With these viruses the loss of infectivity is not closely paralleled by denaturation of the virus protein.

With TMV and potato virus X it is not possible to produce a complete loss of infectivity without substantial denaturation of the protein also occurring. On the other hand, preparations can be made of tobacco necrosis viruses and tomato bushy stunt viruses which have lost all infectivity but which are indistinguishable from infective preparations in their reactions with antisera, their ability to crystallize, and in other physical properties (Bawden, 1950).

Lauffer and Price (1940) concluded that heat denaturation of TMV protein is a reaction of the first order, with an energy of activation in 0.1 *M* phosphate buffer at pH 7 of about 153,000 calories per mole. The rate of heat denaturation was strongly influenced by pH of the solution over the range they examined. For example, at 71°, denaturation proceeded about 3500 times as fast at pH 7.05 as it did at pH 5.77. Infectivity of the virus was lost somewhat more rapidly than virus protein was denatured in their tests.

Hart (1956) used electron microscopy to follow the morphological changes occurring in TMV particles on heating. In the range 80–98° the rod swells at one or both ends. Eventually, the rod is completely converted into a roughly spherical particle of about the same volume as the original rod. Hart estimated that the energy of activation for the conversion of rods to balls is about 150,000 calories per mole (in distilled water), a value in good agreement with that found by Lauffer and Price (1940).

Ginoza (1958) has studied the kinetics of heat inactivation of infectious TMV RNA. The reaction appears to follow a course of first order kinetics. From the moderately low value for the heat of activation of RNA, Ginoza concluded that the loss of infectivity on heating does not involve breakage of a large number of weak bonds. Ginoza found a negative entropy of activation, which he took to indicate that the activated molecule has less degrees of freedom. He suggested that the activated intermediate may be a cyclic phosphate triester, and that the cleavage of only one phosphodiester bond is sufficient to inactivate the RNA molecule.

Turnip yellow mosaic virus is fairly stable to heating in expressed plant sap. The thermal death point under these conditions is 70–75° for 10 minutes heating (Markham and Smith, 1949). If, however, the virus is heated in buffered solution above about pH 6.4, nucleic acid is readily released from the virus protein (Lyttleton and Matthews, 1958). The initial rate of RNA loss increases with increasing temperature between 37° and 50° at pH values above 6.4.

The RNA released from the protein shell after heating for 3 minutes at 45° and pH 7.6 has a molecular weight of about 75,000. Preliminary tests show that most if not all the infectivity of the virus is lost on release of the RNA under these mild conditions.

The behavior in the ultracentrifuge of the protein remaining after escape of the RNA shows that it is not depolymerized into subunits. The sedimentation constant of this material is slightly smaller than the virus protein component containing no RNA. This behavior is consistent with the idea that the RNA, in escaping from the protein, ruptures the shell thereby lowering the symmetry and decreasing the sedimentation coefficient. This protein is not grossly denatured since it is still soluble in water, at least for a time, and its ability to react with specific antiserum is not markedly affected.

The sedimentation behavior of the components formed on heating turnip yellow mosaic virus suggests that the escape of RNA from a virus particle is an all or none event (Lyttleton and Matthews, 1958). If this is so, the data on the proportion of particles which remain intact under various temperatures and pH conditions can only be explained by assuming that the virus particles in a preparation of turnip yellow mosaic virus have a continuous range of stabilities with respect to heating at pH values near 7.0.

The escape of RNA from turnip yellow mosaic virus on heating in buffered solution can be prevented by the addition of sucrose to the virus solution, and there is a linear relationship between sucrose concentration and this protective effect. If it is assumed that the protein shell is impermeable to sucrose, then the reduced loss of RNA in the presence of sucrose might be due to the increased osmotic pressure in the external medium increasing the resistance of the protein shell to rupture from within.

B. *Heating in vivo*

If virus infected plants are held at higher temperatures than normal, the virus may be partially or completely inactivated without killing the plant. Heating of infected plants or parts of plants in water or in air is the only treatment so far discovered which can undoubtedly free fully infected plant material from virus infection. Kassanis (1957) lists 33 viruses which have been inactivated *in vivo* in this way. The most effective temperature has to be determined empirically for each host-virus combination. The margin of safety between inactivating the virus and killing the host is often small.

In countries with high summer temperatures, inactivation of viruses *in vivo* may occur naturally. For example, in parts of India potato tubers kept in ordinary stores may be freed of leaf roll virus, while those kept in cool storage remain 100% infected (Kassanis, 1957; Thirumalachar, 1954). Hitchborn (1956) has shown that a strain of cucumber mosaic virus from an area of the United States with hot summers can multiply in plants at 37°, whereas those from the cool climate of southern England cannot, thus suggesting that natural selection of heat resisting strains may occur.

The relation of heat inactivation *in vivo* to that *in vitro* is not well understood. Those few viruses whose size and shape are known appear to fall into two groups with respect to sensitivity to heat *in vivo* (Kassanis, 1957). Tobacco necrosis, tomato bushy stunt, and carnation ring spot viruses have "spherical" particles and are rapidly inactivated *in vivo*. The first two are known to have a high coefficient of thermal inactivation *in vitro*. Tobacco mosaic virus and potato viruses X and Y have rod-shaped particles, are little inactivated *in vivo*, and have a low coefficient of thermal inactivation *in vitro*. It is by no means established that this distinction between elongated and spherical viruses holds generally. For example, Hitchborn (1957) found a strain of tobacco ring spot virus (which has roughly "spherical" particles) which can multiply at 36° in tobacco plants. TMV may not be completely unaffected in the plant by temperatures around 36° (Kassanis, 1957). When tomato plants systemically infected with bushy stunt virus are kept at 36°, infectivity is lost more rapidly than specific antigen content (Kassanis, 1952).

Harrison's (1956) results with a tobacco necrosis virus in bean show that this virus can multiply at 30° but that some virus is inactivated in the leaves at this temperature. The virus was inactivated more rapidly at 32–35° in the leaf than in expressed sap held at the same temperature. On the basis of these results with tomato bushy stunt and tobacco necrosis viruses (both spherical), Kassanis (1957) suggests that the mechanism of inactivation *in vivo* must be quite different from that occurring *in vitro*, and that at 36° the plant contributes to the destruction of the virus. On the other hand Matthews and Lyttleton (1959) found that the inactivation of turnip yellow mosaic virus in Chinese cabbage plants held at 33° proceeded more slowly than with purified virus *in vitro* held at 33° and pH 6 or 7. A comparison of the properties of turnip yellow mosaic virus which had been inactivated in the plant at 33° with those of normal virus failed to reveal any significant alteration in the ability to crystallize from salt solutions, in ultraviolet absorption spectra, in the rate of release of RNA at pH 7 and 37°, in the ability to combine with antibody protein, or in the proportions of the different virus nucleoproteins that can be isolated by the cesium chloride technique (Matthews and Lyttleton, 1959).

C. *Freezing and Thawing*

Freezing infected leaves, or sap from such leaves, appears to have little effect on many viruses, and is often used as a clarification step in virus isolation, since it renders much of the host protein material insoluble. Some viruses lose infectivity following such freezing and thawing (e.g., alfalfa mosaic and tobacco etch viruses), but this loss may be due to adsorption of virus onto the coagulum of host protein rather than to direct inactivation of the viruses by freezing (Bawden, 1950).

Tomato bushy stunt virus, which is not affected by freezing in the leaf, is inactivated when purified preparations are frozen. On thawing, insoluble inactive precipitates develop (Bawden and Pirie, 1938). The protective effect of the plant sap is accounted for by the observations of Bawden and Pirie (1943) who showed that inactivation was augmented by low pH conditions and reduced by such substances as glucose, protein, and salts.

Loss of infectivity in preparations of tomato bushy stunt virus which have been frozen is usually but not always accompanied by loss of serologic activity and the appearance of a precipitate of denatured protein (Bawden, 1950).

Tobacco ring spot virus is affected by freezing and thawing in a similar way to tomato bushy stunt virus. A large proportion of a purified preparation may be inactivated and the protein denatured by a single cycle of freezing and thawing, but the inactivation can be prevented by the presence of electrolytes, plant pigments, or nutrient broth (Stanley, 1939).

Tobacco mosaic virus appears less affected by repeated freezing and thawing than other viruses, unless the pH is reduced to near 3.0.

III. RADIATIONS

The inactivation of plant viruses has been studied using various ionizing radiations (α-, γ- and X-rays) and ultraviolet light, a nonionizing radiation. The proportion of infective virus particles surviving is an exponential function of the dose (Lea, 1946). The inactivation is independent of the intensity of the irradiation and depends only on the total dose received. The evidence suggests that most of the effect of ionizing radiations on large molecules such as viruses is directly in the virus and not through effects on the medium (Lea, 1946). Since the inactivation of a virus particle by an ionizing radiation can be due to a single ionization within the particle it is possible to calculate the target size from the experimentally determined inactivation dose (Lea, 1946).

A. *X-Rays*

Early work showed that X-rays reduce infectivity of TMV without detectably denaturing the virus protein, as shown by unimpaired serologic activity, anisotropy of flow, or formation of liquid crystals (Bawden and Pirie, 1937).

The dose of X-irradiation required to give 37% survivors of TMV is about 2–3 × 10^5r. This shows that the radiation-sensitive volume of the intact virus particle corresponds fairly closely to that of the virus nucleic acid, and all the evidence now points to the fact that X-rays inactivate the virus by damaging the RNA. The target volume for several spherical viruses also corresponds fairly closely to the volume occupied by the RNA (Buzzell *et al.*, 1956).

The idea that damage to the virus is an all or none event receives support from several experiments. Buzzell *et al.* (1956) found that TMV which had survived a high dose of X-rays was just as stable at 80° as unirradiated virus. Similarly, Lauffer *et al.* (1956) showed that whole TMV that had been heavily irradiated with X-rays has about the same sedimentation rate and intrinsic viscosity as normal virus. The RNA from the virus, however, has a lower intrinsic viscosity than untreated virus, and, therefore, a shorter average particle length. They, therefore, suggested that X-irradiation causes breaks in the virus RNA as it lies within the protein rod, and that the lethal action of X-irradiation is caused by a single break in the RNA of the particle. This idea was confirmed more directly by inactivation studies on infectious RNA isolated from TMV (Ginoza and Norman, 1957). The 37% survival dose for the RNA was 3.0 × 10^5r, a value close to that found for the intact virus. From the results of several experiments they calculated a range of molecular weight for the RNA between 1.4 and 5.4 × 10^6, with a most probable value of 2.7 × 10^6. This is in good agreement with the chemical evidence which suggests a molecular weight of 2.3 × 10^6 for the RNA in a single particle.

B. *Ultraviolet Light*

While the absorption of ionizing radiations is dependent only on the atomic number of the elements in the irradiated material, the absorption of ultraviolet light is very dependent on the chemical bonding in the material. Ultraviolet light is strongly absorbed by the purine and pyrimidine bases found in nucleic acids, there being a maximum of absorption for RNA at a wavelength of about 260 mμ. Proteins also absorb in this region, but much less strongly and with a maximum at about 280 mμ. This type of inactivation has been used mainly for investigating events during the early stages of infection by viruses. The inactivation of viruses by nonionizing radiations has been recently reviewed (Kleczkowski, 1957).

Virus inactivated by ultraviolet light *in vitro* retains full serologic activity and the ability to crystallize, but shows some loss of stability. Oster and McLaren (1950) found that TMV that had been inactivated by ultraviolet light is more readily denatured by heating.

Kleczkowski (1954) (using electron microscopy) showed that a high proportion of irradiated TMV particles had been aggregated side by side to form two-dimensional sheets, while in control preparations most particles were distributed at random. Kleczkowski suggested that irradiation leads to the formation of bonds between neighboring particles.

TMV and tobacco necrosis virus which have been inactivated by ultraviolet light interfere with infection by normal TMV and to a lesser extent with infection by tomato bushy stunt virus and a tobacco necrosis virus (Bawden and Kleczkowski, 1953). In contrast, inactivated tomato bushy stunt virus causes no interference with infection by active bushy stunt virus or TMV. The mechanism of this interference with active virus by inactivated particles is unknown.

With some bacterial viruses, preparations that have been inactivated by ultraviolet light may give rise to new virus if they are inoculated at a high ratio of virus particles to bacterial cells. Bawden and Kleczkowski (1953) could find no evidence for this "multiplicity reactivation" phenomenon with the plant viruses they studied.

Some of the virus particles of tomato bushy stunt, tobacco necrosis, and several other viruses which have been inactivated by ultraviolet light can be reactivated if the plants onto which they are inoculated are exposed to visible light soon after inoculation (Bawden and Kleczkowski, 1953, 1955). Exposing the inactivated virus preparations *in vitro* to visible light does not lead to reactivation. Reactivation presumably occurs because of some light-sensitive mechanism in the host cells. This effect was not observed with TMV. Irradiated preparations of this virus have the same residual infectivity whether plants are placed in the light or the dark after inoculation.

Strains of TMV may show fairly marked differences in their sensitivity to inactivation by ultraviolet light (Siegel and Wildman, 1954). Siegel *et al.* (1956) studied the inactivation by ultraviolet light of a sensitive strain of TMV (U_2), a resistant strain (U_1), and preparations of infectious nucleic acid made from these viruses. The native viruses differed by a factor of five in sensitivity. In contrast, the free nucleic acid from both strains had the same sensitivity as the intact sensitive strain. Different preparations of reconstituted resistant virus had differing sensitivities to ultraviolet light, but some were just as sensitive as the naked nucleic acid. Siegel *et al.* (1956) suggest that the differences in sensitivity between the two strains are due to differences in the nature of the bonding between protein and RNA. Other observations on these strains support the idea that the nucleic acid is more firmly bound to the protein in the resistant than in the sensitive strain. The sensitive strain is also considerably more sensitive to heat denaturation than the resistant strain and the RNA from the sensitive strain is released more easily by the heat-detergent method of Fraenkel-Conrat and Williams (1955).

Few attempts have been made to obtain action spectra for the inactivation of plant viruses by ultraviolet light. The data of Hollaender and Duggar (1936) show no peak of inactivating efficiency at wavelengths between 220 and 300 mμ. The curve rises steadily from about 300 to 250 mμ and then rises much more steeply to 220 mμ. This result might suggest that neither the RNA nor the aromatic amino acids are involved in the inactivation but rather that aliphatic amino acids and peptide bonds are involved. As noted above, intact TMV is more resistant to inactivation than the free RNA with some strains of the virus, while with others both intact virus and free RNA have about the same sensitivity. Presumably with strains of the latter type the action spectrum would bear a fairly close relationship to the ultraviolet absorption spectrum of the RNA. It may be that Hollaender and Duggar used a strain of virus in which the protein had a stabilizing effect on the RNA, and their action spectrum may be in part a reflection of this.

The main value of action spectra has been to indicate the chemical nature of the material involved in inactivation. With TMV the value of such investigations has been lessened by the direct demonstration of the infectivity of isolated RNA. Nevertheless, the determination of fairly precise action spectra for strains of TMV differing in their stabilities to ultraviolet light, and of infectious nucleic acid prepared from such strains might give some information concerning the nature of the bonding between protein and RNA in the virus.

Studies on the inactivation of viruses *in vivo* by ultraviolet light have been mainly limited to investigating changes in the sensitivity in the few hours following inoculation. For the first hour or so after inoculation to bean plants, tobacco necrosis virus is sensitive to ultraviolet inactivation. After about 2 hours, the resistance to inactivation increases and continues to do so up to 15 to 20 hours (Bawden and Harrison, 1955). After 4 hours, the ultraviolet inactivation curve appears to deviate from exponential form, suggesting that some infected cells contain more than one potentially infective particle.

Siegel and Wildman (1956) distinguished four phases in the first 4–7 hours following inoculation of *Nicotiana glutinosa* leaves with the sensitive U_2 strain of TMV. In the first phase, lasting about 2 hours at 20°, the invading particle had about the same sensitivity to inactivation as the virus *in vitro*. In the second phase of about 1½ hours' duration, resistance increases with time. The third phase is a steady level of resistance maintained for about 1½ hours at a level about 2½ times that of phase one. In the fourth phase, a second rise in resistance occurs. The duration of all these phases is shortened when the temperature is raised to 30°. During the first three phases, the survival curve obtained when leaves are given a series of doses of different amounts is exponential. During the fourth phase the survival curve changes from exponential to one of a multitarget type. A curve of this type indicates the presence of more than one infective unit in each incipient local lesion. Siegel and Wildman suggest that this phase corresponds to the first appearance of new virus particles.

Siegel *et al.* (1957) investigated the changes in resistance to ultraviolet irradiation of resistant and susceptible strains of TMV and of infectious nucleic acid prepared from these strains. With whole virus the initial lag period of high sensitivity was 2½ hours for the sensitive strain and 5 hours for the resistant. In contrast, RNA from both strains behaved in the same way, there being very little or no lag before the onset of increasing resistance. This result supports the idea that at an early stage following inoculation with intact virus, the RNA is freed from its coat of protein.

The ability of visible light to reactivate a proportion of virus particles after inoculation of an ultraviolet-irradiated preparation was used by Bawden and Kleczkowski (1955) to study the process of infection by potato virus X in *Nicotiana glutinosa*. They could distinguish three changes of state in the invading particles in relation to reactivation by visible light. In the first state which lasts 15–60 minutes after inoculation, exposure to visible light has no effect, and the number of lesions is not increased. In the second state which lasts 1–2 hours, particles may be reactivated by visible light. If at the end of this period the leaf has not been exposed to visible light, the particles pass into the third state in which they can no longer be reactivated. Bawden (1957) suggests that the first stage may represent the time taken for the particles to enter the host cell or some particular component of the cell. This stage might involve separation of virus RNA from the protein. The sensitive stage may represent a period when free nucleic acid is established in the superficial cells of the leaf. The third stage may begin when plant nucleases irreversibly destroy the nucleic acid of the ultraviolet-inactivated particles.

Although these studies on ultraviolet inactivation *in vivo* provide clear evidence for changes in state of the invading particle, there are difficulties at present in giving a firm interpretation of the results. The major difficulty is that normal leaf constituents absorb ultraviolet light and thus shelter the invading particle from a proportion of the incident radiation. The upper epidermis of *N. glutinosa* transmitted 25–40% of the incident radiation at 254 mμ (Benda, 1955). As an infecting virus particle or its nucleic acid moves more deeply into the epidermal cell, or from the epidermal layer into underlying tissues, it will become increasingly protected from the applied radiation, and the rate of this movement is not known. At the present time it is only an assumption that the virus begins to multiply first in the epidermal cells. If a proportion of the infecting particles becomes increasingly resistant with time, there would be a tendency for the curves—relating fraction surviving to irradiation dose—to become nonlinear in such a way that higher doses give less inactivation than expected from the initial slope. Some of the data given by Bawden and Harrison (1955) show this tendency.

Little is known of the effects of ultraviolet irradiation in plants that have been infected with a virus for some time. In tobacco plants systemically infected with potato virus Y, short exposures to ultraviolet irradiation inactivate most of the virus in the epidermis, but the bulk of the virus in the leaf appears to be unaffected (Bradley, 1954; Bradley and Ganong, 1957). The aphid, *Myzus persicae*, picks up potato virus Y only from epidermal cells. Aphids fed on infected irradiated leaves did not become infected, but mechanical inoculation tests using all the leaf tissues showed that there was little reduction in the total infectious virus in the leaf.

C. *Visible Light*

Since viruses do not absorb light in the visible region they are not greatly affected by it in any direct way. However, the presence of colored "sensitizing" materials in the medium may lead to inactivation.

For example, TMV was inactivated by blue light (wavelength 436 mμ) in the presence of acriflavine (Oster and McLaren, 1950). The inactivation followed first order kinetics. Inactivation occurred in the absence of oxygen but was greatly increased by its presence. By contrast, inactivation by ultraviolet light is unaffected by the presence of oxygen. Combination between acriflavin and the virus appeared to be necessary for inactivation to occur, but the mechanism of the process is not known.

D. *Incorporation of P^{32} into the Virus*

Bacterial viruses containing substantial amounts of P^{32} may become inactivated by the decay of the radioactive element within the virus particle. This is the so-called suicide effect. No detailed study of this type of phenomenon has been made with plant viruses. However, it has been shown that the presence of P^{32} in tobacco leaves at the time of inoculation can reduce the amount of TMV produced and suppress the development of systemic symptoms (Schlegel *et al.*, 1953). An activity of about 100 c.p.m./mg. fresh weight of leaf (or greater) significantly reduced the amount of virus found in systemically infected leaves 10 days after the plants were inoculated. The extent to which this reduction is due to inhibition of infection, rather than inhibition of multiplication, is not known, since the P^{32} was allowed into the leaves almost immediately after inoculation. The presence of P^{32} in older leaves had much less effect on virus development than in younger leaves. Levels of P^{32} giving more than about 200 c.p.m./mg. fresh weight of leaf visibly inhibited terminal growth of stems and roots. Thus, the margin between a dose of P^{32} giving virus inactivation and one damaging the host plant seems to be too small for this type of inactivation to have any value for freeing individual plants from virus infection.

IV. ULTRASONIC VIBRATION

Takahashi and Christensen (1934) first showed that agitation of TMV with high frequency sound waves led to inactivation of the virus. The amount of inactivation increased with time of treatment. Liquid crystalline preparations of TMV immediately lose their birefringence on treatment. The ability to show anistropy of flow decreases and the proportion of small particles increases as the treatment is continued (Kausche *et al.*, 1941). Oster (1947) followed loss of infectivity and change in particle size of TMV (using electron microscopy) with increasing time of treatment with high frequency sound waves. The number of rodlike particles about 280 mμ long decreased with time. At an early stage, particles about 140 mμ long increased in number and then later decreased. In the later stages shorter particles predominated. The decrease in number of particles with a length of 280 mμ was closely associated with the loss of infectivity. These experiments provided supporting evidence for the now generally accepted view that the particle with a length of about 300 mμ is the infective unit of TMV.

It is almost certain that the inactivating effect of high frequency sound waves is due to the breaking of the virus particle. When highly aggregated preparations of TMV were given ultrasonic treatment they disaggregated at first mainly into rods of about 280 mμ in length with an accompanying increase in infectivity, while more severe treatment led to further breaking of the rods, with a loss of infectivity (Newton, 1951). The results of Newton and Kissel (1953) who used higher frequency sound than Oster (1947) suggest that ultrasonic vibration may have a tendency to break the 280 mμ rods of TMV preferentially at a point about 175 to 205 mμ from one end.

The effect of sonic treatment on the RNA within the virus particle has not been investigated. Presumably, when an intact particle is broken, the RNA is also likely to be ruptured at or near the point of cleavage of the protein. Infective RNA prepared from TMV would probably be very sensitive to ultrasonic treatment. The protein part of the virus is not denatured by such treatment in as much as it still combines with TMV antiserum. In fact, Malkeil (1947) showed that ultrasonic treated virus preparations combine with more antibody protein than do normal virus preparations. He suggested that this is due partly to the increased virus protein surface exposed by sonic treatment, and partly to the closer packing of antibody molecules on the smaller fragments. He found no evidence that new antigenic determinants are exposed by sonic treatment. However, the preparations of normal TMV used by Malkeil for the immunization of rabbits probably contained a mixture of intact rods and smaller virus protein particles. This question could well be reinvestigated using antisera prepared with TMV consisting as far as possible only of long rods, and with a preparation of the virus protein subunits prepared by chemical means.

V. Desiccation

Most viruses are inactivated if leaf tissue or sap containing the virus is dried at room temperature. However, McKinney (1947) found that by cutting leaves into small pieces and drying them rapidly at 1°, unstable viruses, such as cucumber mosaic and tobacco ring spot, could retain infectivity for more than a year provided moisture was excluded. This method is now widely employed for preserving cultures of some more unstable viruses.

TMV is outstanding in its resistance to desiccation. However, repeated wetting and drying may lead to a substantial loss of infectivity (Bawden and Pirie, 1937).

Drying over P_2O_5 largely destroys the ability of TMV to form liquid crystalline solutions, and greatly reduces the readiness with which virus solutions show anisotropy of flow (Bawden and Pirie, 1937). Virus inactivated by drying differs from heat inactivated virus in that it precipitates from solution with salt in a similar way to active virus. The protein from virus inactivated by drying is susceptible to attack by trypsin but the rate of hydrolysis is much slower than with heat inactivated virus. On inactivation by drying the RNA is not released from the denatured protein, in contrast to heat inactivation where free RNA is obtained.

Bawden and Pirie (1937) found that there was much less inactivation of purified TMV if drying was carried out slowly. If, however, leaves or sap containing potato virus X are dried slowly, infectivity is lost. When purified preparations are dried, some denatured, nucleic acid-free protein is produced but some active virus remains which can be isolated by centrifugation. The amount of denaturation depends on the pH at which drying is carried out. At pH 6 about half the preparation is denatured, while at pH 4 or pH 8 more than 90% may be lost (Bawden, 1950).

If solutions of purified TMV are sprayed onto a surface held at low temperature (—22 to —15°) and then freeze dried, many of the rods are shattered into small fragments (Rice, *et al.* 1953). The mechanism of this breakage is not known. Many of the virus rods have a very similar appearance to virus which has been partly stripped of protein by treatment with alkali. There is the suggestion of a thin central thread connecting the fragmented pieces of virus rod. In further studies on freeze dried virus, Rice *et al.* (1956) showed that the short pieces of virus rod were not infectious, and they suggest that there was some loss of RNA from freeze dried virus when taken into solution.

VI. HIGH PRESSURES

Many proteins are denatured after being subjected in solution to pressures of 5000 to 10,000 atmospheres for short periods. Basset *et al.* (1938) found that TMV can withstand a pressure of 6000 atmospheres for 45 minutes but that at 8000 atmospheres infectivity, serologic activity, and ability to form paracrystals on the addition of salt are destroyed. Tobacco necrosis virus was more sensitive, losing infectivity above 3000 atmospheres. TMV was almost completely inactivated after a few minutes exposure to a pressure of about 7250 atmospheres (Lauffer and Dow, 1940). Varying amounts of an inactive coagulum of protein are formed. This material has a low phosphorus content suggesting that nucleic acid has escaped from the protein. At 7500 atmospheres the formation of denatured protein appears to follow a first order reaction. Thus, the effects of high pressures on the virus appear to be somewhat similar to those produced by heating.

VII. Aging

A. *In vitro*

Viruses differ widely in the rate at which they become inactivated on standing *in vitro* at room temperature. The main processes involved in this type of inactivation are probably very similar to those concerned in heat inactivation at higher temperatures, but they proceed more slowly. Potato viruses X and Y which lose infectivity and serologic activity at about the same rates when heated *in vitro* also lose these properties at about the same rate on aging *in vitro* at room temperature. On the other hand, tomato bushy stunt and tobacco necrosis viruses may retain full serologic activity for long periods although they lose infectivity after a few weeks or months on standing at room temperatures.

The conditions in the extract in which the virus is stored may have a very marked effect on its stability (Best, 1937). Thus, the results of tests on the inactivation of viruses on standing in expressed sap are difficult to evaluate because of the complexity of the medium. Besides slow thermal inactivation, pH, and oxidation effects, virus may become combined and precipitated with host constituents, enzymes in the sap may attack the virus, and the growth of microorganisms may lead to further destruction of virus. Because of these difficulties most detailed studies on virus inactivation *in vitro* have been made on purified preparations.

B. *In vivo*

Following infection with some viruses such as TMV, the virus content of the plant (as measured by infectivity per unit of tissue) rises to a maximum and then remains at a more or less steady level, although some fluctuations may occur especially in new young growth. However, with some viruses, even in plants grown at normal temperatures, the virus content rises to a maximum and then falls fairly rapidly to a very low level. For example, when young tobacco plants are inoculated with lucerne mosaic virus, the virus content of the plant increases from about the fourth to the twelfth day after inoculation and then decreases rapidly (Ross, 1941). Sap from plants infected for 48 days may have less than 1% the infectivity of plants infected for 12 days. The drop in infectivity is not due to dilution of existing virus by new plant growth. Ross found no evidence for the presence of inhibitors of infectivity in the sap from old plants. The reason for this loss of infective virus *in vivo* is unknown but might be due in part to thermal instability of the virus at normal growing temperatures.

VIII. Hydrogen Ion Concentration

All proteins and ribonucleic acids, including viruses, are disrupted by high or low hydrogen ion concentrations. On the acid side, tobacco ring spot is inactivated below pH 6, potato virus Y below about pH 4.5, tobacco ring spot virus below about pH 4.0, while TMV and tomato bushy stunt virus are stable to pH 2 and below. Most viruses that have been studied appear to be stable up to about pH 8, but different workers have obtained different results with the same viruses (e.g., Bawden and Pirie, 1940a; Best, 1936). Denaturation of potato virus X at pH 7 and 37° is accompanied by loss of RNA from the protein (Bawden and Crook, 1947). Denaturation is prevented, like heat denaturation, by normal sap constituents, and by some other solutions containing proteins.

The degradation of TMV under mildly alkaline conditions has been studied most recently by Harrington and Schachman (1956). They incubated TMV at pH 9.8 at various temperatures and followed the appearance of new components by analysis in the ultracentrifuge. Temperature had a marked effect on the kind of products formed. Of the many components they detected, only two appeared to be true degradation products. One was a protein with a molecular weight of about 10^5 and the other a nucleoprotein rodlike particle about one-third the length of TMV. The other components appeared to be formed from reaggregation of smaller degradation products. They found that some particles with the physical characteristics of TMV are completely resistant to alkali under conditions where most of the virus particles are disrupted. Harrington and Schachman did not characterize the RNA released under their conditions when protein was stripped from the virus. However, it is certain that any RNA released in an infectious state would have been rapidly inactivated. Infectious TMV RNA rapidly loses infectivity on incubation at pH values outside the range pH 5–8 (Fraenkel-Conrat *et al.*, 1957).

Bawden and Pirie (1937) found that treatment of TMV with glacial acetic acid led to a separation of the RNA, as a precipitate, from the virus protein which remained in solution. More recently, the virus protein produced by treatment with cold 67% acetic acid has been shown to have a sedimentation constant of 5.2 S (Fraenkel-Conrat, 1957), which suggests a particle size about the same as the protein subunits produced by alkaline degradation. The nucleic acid obtained by this procedure has very low infectivity compared with preparations made by degradation of virus under alkaline conditions. Dialysis of TMV solutions against 0.02 *M* ethanolamine at pH 10.5 gives a fairly homogeneous protein of low molecular weight (Newmark and Myers, 1957).

When turnip yellow mosaic virus is heated between 37 and 50° in solutions buffered near pH 7, the RNA escapes from the protein shell and infectivity is lost (Lyttleton and Matthews, 1958).

With the only examples that have been studied in any detail (TMV in acid and alkali and turnip yellow mosaic virus near neutrality), changes in hydrogen ion concentration lead to the removal of the virus RNA from its protecting coat of protein. The RNA so exposed is either already inactivated by the treatment or, if still infectious, is in a very unstable condition. It is not yet known whether this exposure of the virus RNA is a general phenomenon in the inactivation of viruses by mild acid and alkali. Treatment of tomato bushy stunt and tobacco necrosis viruses at pH 9 causes a fairly rapid loss of infectivity but serologic activity and ability to crystallize are not affected until the pH is raised to 10 or above (Bawden, 1950). It is not known whether the RNA escapes from these viruses at pH 9, but if it does the proteins must be less affected than those of turnip yellow mosaic virus. With this virus, the protein from which RNA had escaped could no longer be made to crystallize.

IX. Inorganic Compounds

Many of the inorganic substances which inactivate viruses *in vitro* are oxidizing agents or protein precipitants (Stanley, 1935). Some inorganic substances inactivate viruses and at the same time destroy all other characteristic properties of the virus particle. This section deals with some of the treatments which can inactivate viruses *in vitro* without total destruction, and with effects on viruses of inorganic substances applied to the host plant.

A. *Nitrous Acid*

Treatment with nitrous acid under appropriate conditions can destroy the infectivity of potato virus X and TMV preparations without any apparent effect on serologic activity (Bawden and Pirie, 1936, 1937; Stanley, 1936). Still more severe treatment causes destruction of the virus. Stanley (1936) found that mild treatment with nitrous acid eliminated virtually all amino nitrogen from the virus. However, it may be that alteration of these groups on the virus protein plays little part in the loss of in fectivity. Structural changes in the RNA would almost certainly be of much greater significance.

B. *Hydrogen Peroxide*

Careful treatment with hydrogen peroxide can also give noninfectious virus preparations with full serologic activity. TMV is more resistant than potato virus X to complete denaturation by this reagent.

C. *Iodine*

The reaction of TMV with iodine under appropriate conditions leads to the oxidation of —SH groups to —S—S— in the virus protein, but not to loss of infectivity. Under somewhat more severe conditions (a higher concentration of iodine or higher temperature of reaction) the tyrosine groups are converted to diiodotyrosine and infectivity is lost (Anson and Stanley, 1941). Presumably, inactivation is not due directly to a change in the tyrosine residues but to some other changes going on under the same conditions leading to inactivation of the virus RNA.

D. *Salts*

Neutral salts appear to have little permanent direct effect on most viruses that have been tested, although the presence of such salts in the inoculum may affect the number of local lesions produced. However, tobacco ring spot is inactivated by standing at 4° in 10% ammonium sulfate solution. The inactivation is accompanied by loss of RNA from the protein (Stanley, 1939). Salts reduce infectivity of viruses with elongated particles by inducing end to end aggregation of the particles. However, the infectivity lost in this way may be restored by such treatments as very mild alkali. Rothamsted tobacco necrosis virus is made noninfective by certain salts (e.g., *M* NaCl). The virus is not disrupted and its serologic activity is unaffected (Bawden and Pirie, 1945).

TMV is split at room temperatures into denatured protein and free RNA by molar solutions of $Sr(NO_3)_2$ and to a lesser extent by $SrCl_2$, Ba $(NO_3)_2$, Ca $(NO_3)_2$ (Pirie, 1954). Sr $(NO_3)_2$ precipitates tomato bushy stunt virus but does not separate the RNA from the virus protein. No infectivity measurements were made on the RNA produced but residual infectivity, if present, would presumably have been rapidly lost.

The stability of infective preparations of TMV RNA is markedly affected by ionic conditions in the medium in which it is incubated (Fraenkel-Conrat *et al.*, 1957). Most of the infectivity is lost on incubation at 36° for one hour in all salt solutions tested at 0.1 *M* concentration, but in 0.01 *M*. buffers it was comparatively stable over the range pH 4.6–9.2. In very low salt concentrations (0.001 *M*), even less infectivity was lost. On the other hand, very high salt concentrations (*M*–2*M*) had the most marked protective effect, and in some experiments caused an increase in infectivity over RNA which had been stored in a frozen state. It was suggested that this protective effect was due to aggregation and precipitation of the RNA.

Zinc sulfate or zinc chloride at concentrations around 1/2000 greatly reduces the number of local lesions produced by TMV in *N. glutinosa* leaves floated on solutions of the compound (Weintraub *et al.*, 1952). The treatment is effective when applied 6 hours after inoculation. Zinc sulfate (1/500) had no detectable effect *in vitro* on the infectivity of TMV. Yarwood (1954) showed that when bean leaves which had been inoculated with TMV are dipped in 0.01 to 0.03% solution of zinc sulfate for 10 minutes, beginning 10 minutes after inoculation, the number of local lesions produced is greatly increased campared with water controls. However, when Yarwood used the host and method of application of the compound used by Weintraub *et al.*, (1952), he confirmed that zinc treatments reduce the number of local lesions. The reason for these two different effects is not apparent. When sprayed on bean leaves before inoculation, certain metal nitrates (Cs, Ba, Al) increase the number of local lesions produced by a tobacco necrosis virus. Others have little effect, but zinc nitrate causes a striking reduction (Matthews and Proctor, 1956).

Stoddard (1947) stated that the virus causing X disease of peach could be inactivated in living peach buds by soaking buds in solutions of zinc sulfate. Rumley and Thomas (1951) treated twelve mosaic-infected carnation cuttings with a dilute solution of zinc chloride. About half the plants developing from these cuttings appeared to be free of virus. However, treatment with zinc salts does not appear to have become an accepted practice for freeing peach and carnation material from these viruses.

X. ORGANIC COMPOUNDS

In this section, the *in vitro* and *in vivo* effects of some organic compounds on viruses are summarized. The division between this section and the next two is to some extent arbitrary. In general, simple synthetic substances are dealt with here, and metabolites and antimetabolites in Section XI. The effects of more complex and often unidentified substances in plant extracts are discussed in Section XII.

A. *Urea*

Urea is a well-known protein denaturant and its effects on viruses have been widely studied (Bawden and Pirie, 1937; Stanley and Lauffer, 1939). Bawden and Pirie (1940a) showed that denaturation of four different viruses by urea is closely linked with loss of infectivity and serologic acitvity. With each virus, the reduction in infectivity is roughly proportional to loss of serologic activity. The inactivation of potato virus X and TMV results in separation of RNA from the protein, and the products of denaturation are soluble in urea solutions. With tomato bushy stunt and tobacco necrosis viruses the RNA remains with the protein and the denatured material is insoluble in urea.

B. *Formaldehyde*

Formaldehyde has been widely used as an inactivator for animal viruses and bacteria in the preparation of vaccines. It is a convenient reagent for preparing noninfectious virus without loss of serologic activity. Ross and Stanley (1938) stated that TMV preparations which have been inactivated by treatment with formaldehyde at pH 7 can be partially reactivated by dialysis at pH 3. Inactivation is accompanied by a decrease in amino groups and a decrease in the number of groups reacting with Folin's reagent at pH 7.7 (probably the indole ring of tryptophan). They suggested that reactivation is accompanied by an increase in these groups. Kassanis and Kleczkowski (1944) were unable to confirm the reactivation of virus, nor did they find any correlation between inactivation and groups giving the Van Slyke test for amino nitrogen or reacting with Folin's color reagent. They found that TMV is inactivated by 2% formaldehyde at pH values between 3 and 7.5, the rate being slowest at pH 3.5. Inactivation could be stopped at any stage by dilution or dialysis.

The possibility that the RNA might be involved in the inactivation of TMV by formaldehyde was suggested by Fraenkel-Conrat (1954) on the basis of spectral changes he observed after the reaction of TMV with the compound and because after treatment of intact virus he found about 0.8% formaldehyde in the RNA and only 0.05% in the protein.

From the results of kinetic studies on the loss of infectivity of TMV on treatment with formaldehyde, Cartwright *et al.* (1956) concluded that inactivation of a single virus particle could be caused by reaction with one molecule of formaldehyde. They suggested that the groups most likely to be involved in the inactivation are the beta hydroxy groups of serine and threonine, the hydroxy groups of ribose, and the amino groups on the purine and pyrimidine rings.

Infectious RNA prepared from TMV was found to be very sensitive to formaldehyde, being inactivated by less than 1/100th the concentration required to inactivate intact virus (Staehelin, 1957). The greater part of the formaldehyde is bound onto RNA in some reversible form and is lost on dialysis. A smaller amount is more firmly bound and the relative amount of this material increases with the time of reaction. Under conditions giving partial inactivation of an RNA preparation, only one formaldehyde molecule is bound per several hundred nucleotides. There was some indication that cross-linking of RNA molecules may occur following reaction with formaldehyde (Staehelin, 1957).

C. *Carbon Dioxide*

Raising the level of carbon dioxide in the atmosphere reduces the susceptibility of bean plants to tobacco necrosis virus (Kalmus and Kassanis, 1944). The treatment is effective if applied up to 2 hours after inoculation, but at 4 hours has no effect. The mechanism of this inhibition is unknown. Placing tobacco plants in 50% carbon dioxide had no effect on their susceptibility to potato virus Y introduced by aphids (Bradley and Ganong, 1957).

D. *Organic Acids*

Potato virus X loses almost all infectivity, serologic activity, and other characteristic properties when incubated with sodium citrate at pH 7 and 37° (Bawden and Crook, 1947). In contrast, Rothamsted tobacco necrosis virus loses infectivity but retains full serologic activity and the ability to crystallize when exposed to sodium citrate at pH values near neutrality (Bawden and Pirie, 1950). The presence of citrate and probably some other organic acids may well be a factor leading to the inactivation of certain viruses in plant extracts.

Such acids may also play a part in limiting the establishment and multiplication of some viruses. A range of organic acids at concentrations around 0.1 *M* when sprayed on the leaves of bean plants before, or up to about 2 hours after inoculation, greatly reduces the number of local lesions produced by a tobacco necrosis virus (Matthews and Procter, 1956).

Substances like citric and succinic acids are known to form chelate complexes with certain metal ions. The inhibitory effect of such acids could be annulled by the application of salts of certain metals such as calcium and magnesium. These effects suggest that the tobacco necrosis virus like some bacterial viruses may require the presence of certain metal ions for successful establishment. However, Wiltshire (1956a, b) found no correlation between concentration of various organic acids (including citric acid) in bean leaves, and changes in the susceptibility of the plants to tobacco necrosis virus.

E. *Organic Solvents*

The protein of turnip yellow mosaic virus is split from the RNA and denatured by 30% ethanol at pH 7 and room temperature (Markham and Smith, 1949). With purified TMV at pH 7 in salt-free solution there is no precipitation of virus until the ethanol concentration is carried above 80%. Denaturation of virus proceeds slowly in 80% ethanol (Bawden and Pirie, 1937). The presence of salt lowers the ethanol concentration at which virus precipitation occurs.

F. *Surface Active Agents*

Sodium dodecyl sulfate under mildly alkaline conditions can cause the separation of virus protein from RNA not only with TMV and potato virus X, but also with tomato bushy stunt virus (Bawden and Pirie, 1940b; Sreenivasaya and Pirie, 1938). Under suitable conditions, the products formed by the action of sodium dodecyl sulfate remain soluble in dilute salt solutions, although infectivity is lost. Treatment of TMV with sodium dodecyl sulfate under more closely defined conditions (pH 8.5–8.8 at 50° for a few minutes) has been found to yield RNA preparations which retain about 1 to 5% of the infectivity of undegraded TMV (Fraenkel-Conrat *et al.*, 1957).

G. *Some Other Organic Substances*

Bawden and Pirie (1940b) studied the effect of urethane, guanidine, pyridine, picoline, lutidine, aniline, phenol, salicylic acid, and benzoic acids on several viruses. All the substances inactivated TMV, potato virus X, and tomato bushy stunt virus in neutral solution. Dilute solutions of these substances often acted merely as precipitating agents without causing irreversible inactivation. As with urea, inactivation of TMV and potato virus X with these compounds leads to separation of the RNA from virus protein but no such separation occurs with tomato bushy stunt virus. Potato virus X is most sensitive and tomato bushy stunt virus least sensitive to denaturation by these agents. None of these inactivators gives an increased rate of denaturation on cooling below 20° as occurs with urea.

H. *Synthetic Polypeptides*

Synthetic polypeptides built up from lysine inhibit the infectivity of TMV (Stahmann *et al.*, 1951). Such polypeptides combine with and precipitate the virus from solution. Free lysine causes no precipitation (Burger and Stahmann, 1951). The inhibitory effect on TMV increased with increasing average chain length of the synthetic polypeptide.

Burger and Stahmann (1951) suggested that the basic peptides combine with the virus protein by forming numerous ionic bonds between the oppositely changed virus and peptide.

I. *Dyestuffs*

Takahashi (1948), using detached *N. glutinosa* leaves, found that the amount of TMV produced was reduced when leaves are floated on solutions containing malachite green. Similarly, growth of virus tumors from *Rumex acetosa* L. is inhibited by malachite green, methylene blue, and crystal violet (Nickell, 1951). Of 16 shoot tips from virus X-infected potatoes cultured on agar containing malachite green (3 p.p.m.) one was subsequently found to be free of virus (Norris, 1953). Norris attributed the absence of virus in this shoot to the treatment, but it seems probable that the dye may have had little influence. Thompson (1956, 1957) found that potato plants could be obtained free of virus Y merely by excision of the apical region of etiolated shoots, and it may be that occasional shoots can be free of virus X in a similar way.

J. *Acridine Derivatives*

Trypaflavin depressed multiplication of TMV in tobacco leaves and it also reduced the necrotic reaction of *N. glutinosa* to TMV. *In vitro* trypaflavin precipitates TMV from solution but the virus is still infectious after dialysis (Richkov and Smirnova, 1948). Acriflavine and 5-aminoacridine have no marked effect on TMV increase in leaf discs (Schlegel and Rawlins, 1954).

XI. METABOLITES AND ANTIMETABOLITES

A. *Vitamins, Coenzymes, and Related Compounds*

Very few vitamins or related compounds have been tested as virus inhibitors. Richkov *et al.*, (1946) found that thiamine causes no inactivation of TMV *in vitro,* but reduces the amount of virus produced in tobacco leaves cultured on solutions of the compound, and the number of lesions on leaves. A number of other vitamins and related compounds had little or no effect on several viruses (Matthews and Smith, 1955; Schlegel and Rawlins, 1954; Schneider, 1954).

B. *Plant Growth Regulators and Related Compounds*

Limasset and Cornuet (1949) drew attention to the association in tobacco meristems of a low content of TMV with a high concentration of auxin. Augier de Montgremier and Morel (1948) obtained results suggesting that less TMV developed in tobacco tissues with a high content of naphthalene acetic acid. Various compounds of the plant growth regulator type have been shown to reduce the virus content of plants or to mask symptoms of disease (Kutsky, 1952; Kutsky and Rawlins, 1950; Limasset *et al.*, 1948; Locke, 1948; Nichols, 1952; Nickell, 1950).

C. *Amino Acids*

According to Richkov (1951), necrotic lesions of TMV in *Nicotiana glutinosa* were inhibited by norleucine, taurine, glutamic acid, threonine, lysine, and cysteine, and the multiplication of TMV in tobacco by the last four. Similarly, Schlegel and Rawlins (1954) reported that TMV in tobacco was inhibited by L-isoleucine and ethionine, but not by D-isoleucine or D,L-benzoylalanine.

D. *Analogues of the Natural Purines and Pyrimidines*

In view of the vital role of the nucleic acid in virus multiplication, there is particular interest in the effects of compounds which may interfere with its synthesis or functioning. Many synthetic analogues of adenine, guanine, cytosine, and uracil have been tested for inhibitory effects on plant viruses (Kurtzman *et al.*, 1957; Matthews and Smith, 1955; Schneider, 1954). Since the effects of purine and pyrimidine analogues on viruses and other biological materials have been recently discussed in detail (Matthews, 1957, 1958b; Matthews and Smith, 1955; Smith and Matthews, 1957), we will consider only a few of the main points here.

Interest has centered on two compounds—8-azaguanine, in which the carbon 8 of guanine is replaced by nitrogen, and 2-thiouracil in which the hydroxyl substituent in position 2 of uracil is replaced by sulfur. Both these compounds inhibit the development of certain viruses (Bawden and Kassanis, 1954; Commoner and Mercer, 1951, 1952; Matthews, 1953; Mercer *et al.*, 1953; Nichols, 1953, 1954; Russell and Trim, 1957) when plants are sprayed with solutions of the compounds before or shortly after inoculation, or when inoculated leaves are floated on solutions containing the compounds. Both analogues may cause some damage to the plant tissues, but 2-thiouracil is much more toxic than 8-azaguanine.

Both analogues are incorporated into the RNA of TMV when leaves in which this virus is multiplying are treated with the compounds (Jeener and Rosseels, 1953; Matthews, 1954, 1956). 8-Azaguanine may replace about 3% of the guanine residues, while 2-thiouracil is incorporated to an extent equivalent to about 3 to 10% of the virus uracil.

The distribution of 2-thiouracil in TMV RNA relative to uracil has been studied (Mandel *et al.*, 1957). The analogue appears to occupy certain terminal positions in the RNA chains with high frequency. However, this problem requires reinvestigation since certain apparent end groups in TMV RNA may be due to contamination (Reddi *et al.*, 1957).

Preparations of TMV and turnip yellow mosaic virus containing 8-azaguanine are less infectious than normal virus when compared on an equal RNA basis (Matthews, 1954, 1955). The ratio of virus protein and nucleoprotein particles produced in Chinese cabbage plants is constant under a wide range of conditions (Matthews, 1958a). Treatment of the plants with 8-azaguanine has no effect on this ratio (Matthews, 1955). The effects of 2-thiouracil incorporation on the biological activity of TMV are puzzling, and require further investigation (Jeener, 1954, 1957; Matthews, 1958b).

Another uracil analogue, 5-fluorouracil, is incorporated into the RNA of TMV formed in tobacco leaf discs floated on solutions of the analogue (Gordon and Staehelin, 1958). The analytical data suggest that about one-third of the uracil residues are replaced by 5-fluorouracil under conditions where the yield of virus is reduced by 50%. No detailed results on the infectivity of virus containing the analogue have yet been published but Gordon and Staehelin (1958) state that such virus is as infective as normal virus. The simplest explanation for the virus-inhibitory effects of compounds such as 8-azaguanine and 2-thiouracil is that they lead to the production of virus RNA which is incapable of further replication. This would be sufficient to explain the observed effects on virus multiplication. 2-Thiouracil treatment causes a marked rise in the levels of certain amino acids in tobacco leaves, and this is associated with a decrease in protein (Porter and Weinstein, 1957). This effect could also be explained on the assumption that synthesis of host protein is dependent on functional RNA and that thiouracil incorporation interferes with this function.

However, it may well be that compounds such as 8-azaguanine and 2-thiouracil also interfere in other ways with processes involved in virus multiplication. For example, they might become incorporated into soluble nucleotides to form analogues of compounds such as guanosine triphosphate and uridine diphosphate and these more complex analogues may interfere with normal processes.

The different effects on virus multiplication produced by 8-azaguanine or 2-thiouracil in different host species may well depend on the extent to which the analogues are detoxicated by conversion to inert compounds, or synthesized into more complex inhibitors. As far as effects on different viruses are concerned, these compounds undoubtedly show some specificity. For example, if *Nicotiana glutinosa* plants suitably sprayed with 8-azaguanine are inoculated with a mixture of lucerne mosaic virus and potato virus X, the latter virus moves systemically as in untreated plants while lucerne mosaic virus multiplies to a limited extent in the inoculated leaves and does not move into the rest of the plant. The basis for this specificity is quite unknown.

As the leaves on a normal plant approach senescence they usually become less susceptible to infection with a virus and multiplication in the leaf may be limited. With a virus such as lucerne mosaic in *Nicotiana glutinosa,* it may be that 8-azaguanine merely delays multiplication in the inoculated leaves long enough for the natural process of senescence to take over and permanently limit the spread of the virus. A similar situation is probably involved in the suppression by 2-thiouracil of a systemic necrotic reaction to TMV in certain tobacco varieties (Holmes, 1955).

XII. Substances from Organisms

Extracts or fluids from many animals and microorganisms, as well as from higher plants, have been shown to inhibit infection by plant viruses. Many of the organisms known to give inhibitory extracts have been listed in a recent review (Bartels, 1955) and most of these will not be referred to here.

A. *From Animals*

A variety of fluids from animals have been found to inhibit infection by plant viruses (Bawden, 1950; Johnson, 1941; Smith, 1941). The effects of certain enzymes and of normal and immune serum from rabbits have been studied in most detail.

1. *Rabbit Sera*

When viruses are mixed either with normal serum from nonimmunized rabbits or with specific antisera prepared against them in rabbits, the infectivity of the preparation is reduced. Chester (1934) suggested that the normal serum acts on the host plant while a specific antiserum acts directly on the virus, but later work suggests that both effects of antiserum are due to direct action on the virus. Kassanis (1943) found that with fresh sera the nonspecific effect was great and largely obscured the specific neutralization, while in old sera the specific effect predominated. The virus is not destroyed by mixing with normal serum or antiserum. With TMV, some infectivity is regained when mixtures are diluted, and enzymatic hydrolysis of antibody protein leads to release of active virus from specific precipitates (Bawden and Pirie, 1937).

Rappaport and Siegel (1955), using an antiserum of high antibody content were able to distinguish clearly between the specific effect of antibody and nonspecific serum constituents on TMV. Antiserum caused about 100 times more inactivation than normal serum. The relationship between percentage survival and concentration of serum differed for antiserum and normal serum. The inactivation by antiserum was very rapid. They found that half the activity of a TMV solution was lost 15 seconds after mixing with antiserum, but there was no further loss after 15 minutes incubation.

There is no evidence that the RNA of viruses plays any part in the combination between antibody protein and the virus. Infective RNA from TMV is little affected by TMV antiserum under conditions where the whole virus is inactivated (Gierer and Schramm, 1956). Fraenkel-Conrat *et al.*, (1957) found that both normal and TMV antiserum frequently cause some inactivation of infective RNA. When γ-globulin fractions of both antiserum and normal serum were tested, RNA activity was not appreciably affected under conditions where whole TMV was largely inactivated. Their results strongly suggest that antibody protein inactivates the virus directly, and not indirectly through some effect on the host plant. It may be that the bonding of antibody to the virus protein prevents effective release of RNA from the protein at an early stage in virus establishment.

2. *Pancreatic Ribonuclease*

When pancreatic ribonuclease is mixed with fairly concentrated solutions of TMV in the absence of salt, a virus-enzyme complex separates from solution in the form of long, fiber-like particles containing about 14% ribonuclease (Loring, 1942). This virus-enzyme complex is noninfective, but can be readily dissociated by dilution to give fully active virus. Loring obtained no evidence for any enzymatic breakdown of the virus by ribonuclease, since there was no increase in the degree of inactivation on incubation of the virus-enzyme mixture. He suggested that inactivation of the virus was due, at least in part, to the combination of enzyme with virus. Combination between virus and enzyme in neutral solution would be expected since the isoelectric point of TMV is about pH 3.5 while that of ribonuclease is about pH 8.0. A number of observations suggest that the combination between enzyme and virus may not be of major importance in causing the inactivation.

For a given mixture of enzyme and virus Loring (1942) found a much greater inactivation when infectivity tests were made on *Phaseolus vulgaris* compared with *Nicotiana glutinosa*. This might indicate (a) some effect of ribonuclease on the host plant, or (b) that *N. glutinosa* is more effective than *P. vulgaris* in splitting the noninfective complex.

Casterman and Jeener (1955, 1957) infiltrated tobacco leaves with a dilute solution of ribonuclease. Infiltration before inoculation blocked development of TMV on tobacco. Infiltration after inoculation was progressively less effective up to 2 hours, and at 6 hours had no effect. In leaves that were merely dipped in an enzyme solution for one hour and then washed off before inoculation, virus multiplication occurred normally.

Reversible oxidation of ribonuclease by hydrogen peroxide suppressed both its enzymatic activity and its ability to inhibit TMV infection, but left its power to combine with TMV unimpaired. Casterman and Jeener (1957) suggested, as an alternative to inactivation through complex formation, that TMV might pass through an initial stage during which its RNA is susceptible to the action of ribonuclease. This idea receives support from the fact that TMV RNA stripped of protein is capable of infection, and that such naked RNA is extremely susceptible to inactivation by ribonuclease (Fraenkel-Conrat *et al.*, 1957; Gierer and Schramm, 1956). Concentrations of the enzyme 1000-fold higher than those inactivating RNA had no effect on intact TMV. Pancreatic deoxyribonuclease and trypsin both inactivated TMV RNA at concentrations 100–1000 times higher than that at which ribonuclease was active. These activities were stable to heating at 90°. For this reason, the activity of deoxyribonuclease and trypsin preparations was attributed to contamination with small amounts of ribonuclease.

Infectious RNA prepared from TMV is also extremely susceptible to enzymes (presumably nucleases) which are released when leaf cells are broken during the inoculation process. By dipping an inoculation pad or spatula that has already been used to rub a leaf into an inoculum containing free infectious RNA, sufficient enzyme may be introduced to cause rapid inactivation.

3. *Proteolytic Enzymes*

Trypsin has no proteolytic action on some viruses (e.g., TMV) while it degrades the protein of others (e.g., potato virus X). Stanley (1934) showed that loss of infectivity occurs immediately on mixing TMV and trypsin. Virus activity is lost at pH values where trypsin is not proteolytically active, and no further loss of activity occurs when conditions are made suitable for proteolysis. Stanley showed that infectivity could be regained by diluting the virus enzyme mixture, or by digestion with pepsin. Stanley (1934) suggested that trypsin affects the resistance of the host and has no direct effect on the virus.

The infectivity of potato virus X preparations is reduced immediately upon the addition of trypsin, and there is a second drop of infectivity if conditions are suitable for proteolysis (Bawden and Pirie, 1936). This second drop is accompanied by loss of serologic activity and destruction of the virus. Both TMV and potato virus X combine with trypsin, but the virus-enzyme complex is readily broken by dilution (Kleczkowski, 1944). Trypsin is only about 1/100th as effective as ribonuclease in inhibiting these viruses. Ribonuclease is known to be a contaminant in many preparations of trypsin and it may be that the presence of this enzyme plays an important part in the inactivation of viruses by trypsin preparations.

Pepsin hydrolyzes potato virus X under suitable conditions leading to loss of infectivity and serologic activity (Bawden and Pirie, 1936). This enzyme has little effect on infective TMV, and does not combine with it (Kleczkowski, 1944). Denatured TMV protein is, however, hydrolyzed by the enzyme.

4. *Extracts of Insects*

Extracts from the macerated tissues of many insects contain potent inhibitors of infection (Smith, 1941). There seems to be no association between the presence or absence of such inhibitors and the ability of an insect to transmit a particular virus. When the virus is taken in by an intact insect there is no reason to suppose that it would come in contact with substances liberated in macerated tissues. None of the inhibitors from insects appears to have been identified. The ability of juice from a clover leaf hopper to inhibit infection by TMV was not affected by dialysis but was destroyed by heating and strong acids or bases. This suggests that the inhibitor may have been a protein (Black, 1939).

B. *From Microorganisms*

Most of the antibiotics developed for use against bacterial diseases have been tested against plant viruses, but no substantial effects have been obtained with any of them (Beale and Jones, 1951; Kirkpatrick and Linder, 1954; Leben and Fulton, 1952; Manil, 1947).

So far there has been little systematic search for new antibiotics specifically active against plant viruses. A recent report (Gray, 1957) describes preliminary results with one such compound which was named "cytovirin." This material sprayed on the leaves near the time of inoculation completely prevents local lesion production by Southern bean mosaic virus on Pinto bean plants and by TMV on *Nicotiana rustica.* Development of systemic disease was also delayed or prevented.

Gupta and Price (1950, 1952) tested the culture filtrates from 49 fungal species and found that filtrates from *Trichothecium roseum* and *Neurospora sitophila* were the most inhibitory. Bawden and Freeman (1952) have shown that two substances are involved. Most of the inhibition is due to a polysaccharide which contains D-galactose as the main component sugar. This polysaccharide does not precipitate TMV nor combine with it to form a soluble complex. It is effective if sprayed on the leaves before inoculation but not afterward.

The other inhibitor was identified as trichothecin (an ester of isocrotonic acid with the ketonic alcohol trichothecolone) which is also an antifungal substance. This inhibitor was effective if sprayed on the leaves not more than 2 days before or not more than one day after inoculation. In contrast to trichothecin, trichothecolone and acetyltrichothecolone were more effective in preventing infection of *N. glutinosa* than of beans. These two derivatives damage *N. glutinosa* but not bean leaves, while the reverse situation holds with trichothecin. The relative efficiency of the inhibitors depends on the species of host plant inoculated rather than on the virus. This result might suggest that the inhibitors affect the host plant directly rather than the virus. However, in the absence of any evidence concerning the action of the host plant on the substance applied it is difficult to draw any definite conclusion about the mode of action of these substances. The compounds applied to the leaf might be converted by particular host species either to inert substances or to active inhibitors acting directly on the virus.

Bradley and Ganong (1957) found that spraying tobacco plants with concentrations of trichothecin which caused no obvious damage could protect most plants against infection by aphid-borne potato virus Y.

C. *From Higher Plants*

The sap from many higher plants contains substances which inhibit infection by plant viruses. The unsuspected presence of such inhibitors has often led to mistaken ideas concerning the host range and transmissibility of particular viruses. Where a virus is known to infect only host species containing high levels of inhibitor, its presence may greatly complicate the isolation and purification of the virus. In general, inhibitors present in sap from a particular species are effective only when the virus is being inoculated to other host species. For example, cucumber mosaic virus can be transmitted mechanically from one *Phytolacca* plant to another, but not from *Phytolacca* to tobacco. Sugar beet mosaic and cucumber mosaic viruses can be transmitted mechanically from infected to healthy beet, but not from beet to tobacco, when undiluted sap is used; but cucumber mosaic can be transmitted if the beet sap is diluted 1/10 with water (Bhargava, 1951). In a systemic study with several plant viruses and host species, Gendron and Kassanis (1954) found that the extent to which inhibitors decrease the number of local lesions produced is independent of the virus tested and depends only on the species on to which the virus is inoculated.

Sap from species of *Phytolacca* is one of the most potent inhibitors known from higher plants and is one of the few whose chemical nature has been determined. Kassanis and Kleczkowski (1948) isolated the inhibitor from sap of *P. esculenta* and found it has the composition expected for a glycoprotein. The glycoprotein has an isoelectric point near pH 7, and can combine with TMV. Under appropriate conditions it precipitates the virus as paracrystalline threads, and can also precipitate tomato bushy stunt virus. The glycoprotein causes an immediate reduction in infectivity when mixed with TMV and several other viruses. The infectivity of such mixtures are regained on dilution. Loss of infectivity is not associated with a fixed combining ratio between virus and glycoprotein. On an equal weight basis the glycoprotein preparations are about as effective as ribonuclease in inhibiting TMV.

The mechanism of virus inhibition by the glycoprotein is not known. It is possible that the combination with virus plays some part, but some other proteins such as clupein and globin which can combine with and precipitate TMV have very much less inactivating power (Kleczkowski, 1946).

There is no evidence that a plant species which gives a highly inhibitory sap, is in any way protected from virus infection in nature. Many rosaceous species are commonly infected with a range of viruses, and yet extracts of these plants are usually potent virus inhibitors. Water extracts from macerated leaves, stems, or roots of strawberry plants contain sufficient tannins to precipitate all the plant proteins in the extract (Bawden and Kleczkowski, 1945). If this precipitate is removed, the supernatant fluid still contains enough tannin to precipitate added TMV and render it noninfective for *Nicotiana glutinosa*. Unlike many other inhibitors of infectivity, tannic acid has no effect when rubbed on the leaves immediately after inoculation (Thresh, 1956) and a treatment with the compound before inoculation has little effect if the leaves are washed with water between treatment and inoculation with virus. Tannins do not always give maximum inhibition immediately after mixing with the virus. There appears to be no specifity in their action for they inhibit all the viruses that have been tested, irrespective of the host species used for inoculation. Thus, it appears likely that tannins may cause inhibition of infection by acting directly on the virus. Thresh (1956) discusses various ways in which the inhibitory effects of tannins may be partially avoided.

Chloroplast suspensions prepared from various plants including species of *Nicotiana* may reduce infectivity when mixed with TMV preparations (Chiba and Tominaga, 1952). The inhibitory activity is destroyed by heating.

Sill and Walker (1952) studied the distribution of an inhibitor of cucumber mosaic virus in extracts of cucumber plants. They found it in all parts of the plant except possibly the corolla. Extracts from the green areas of leaves showing mottled symptoms due to the virus are much more inhibitory than yellow areas which contained high concentrations of infective virus. There is a rough correlation between the amount of chlorotic tissue (in which inhibitor content is low) and the degree of resistance of the cucumber variety. However, there is no direct evidence that the presence of the inhibitor in the extracts has anything to do with the resistance of the plant to the virus.

Sap from spinach plants contains at least two kinds of substance which can inhibit infection by a number of viruses (Kuntz and Walker, 1947). Extracts from varieties of spinach which are resistant or susceptible to cucumber mosaic contained about the same amount of an inhibitor for the virus when tests were made on virus in *Nicotiana glutinosa* sap inoculated to cowpea (Pound and Cheo, 1952).

Most viruses are not transmitted through the seed, and it has been suggested that this may be due to the presence of inhibitors. For example, Cheo (1955) found that in the sepals, petals, and young fruits of bean plants infected with Southern bean mosaic virus there is as high a concentration of virus as in the leaves. As the seed matures the virus content of the embryo increases while that of the seed coat and pod decreases. As dehydration of the seed occurs, virus in the embryo is rapidly inhibited. Crude extracts of bean seed contained a heat-stable inhibitor of the virus, and there was more active inhibitor in old than immature seeds.

Crowley (1955) found virus inhibitors in tobacco and cucumber seeds which are probably protein in nature. He considered that these inhibitors have their effect only on the plants used to test for the presence of virus, and are probably of no significance in preventing virus transmission through the seed. Experiments on the significance of inhibitors in plant extracts may be further complicated by the presence of both inhibitory and stimulatory substances in the same extract (Benda, 1956).

Virus inactivators present in the host tissues or appearing when the tissues are disrupted may complicate studies on the properties of purified virus preparations. This possibility is well illustrated by the system in tobacco leaves which can inactivate Rothamsted tobacco necrosis virus

(RTNV) (Bawden and Pirie, 1957). The ratio of infectivity to amount of specific antigen contained in purified preparations of RTNV varies with different preparations. Bawden and Pirie found that this variation can be largely accounted for by the presence of an inactivating system in tobacco leaves. This system is present in the material which sediments from leaf sap centrifuged between 4000 to 8000 *g*. Inactivation by this material requires air and is inhibited by azide, but when the sedimented material is kept at 0° for several hours, a low molecular weight substance separates from it. This low molecular weight material inactivates the virus whether or not air or azide are present. This inactivating material was not characterized chemically, but Bawden and Pirie point out the analogy between this inactivator and chelating agents—such as citrate—which also inactivate RTNV. Various aldehydes are also active at low concentrations as inactivators of this virus. Preparations of RTNV inactivated by the system from tobacco leaves retain serologic activity and resemble infective preparations in all other properties studied.

The material sedimenting from healthy tobacco leaves or from leaves infected with TMV is less effective in inactivating RTNV than material from leaves infected with this virus or tobacco ring spot virus. The sediments inactivate tobacco ring spot virus but not TMV. The significance of the inactivating system *in vivo* is quite unknown, but with *in vitro* experiments it can lead to unexpected results. Thus, RTNV prepared by centrifuging freshly expressed sap at 0° may be less infectious than material prepared by apparently less gentle methods.

Schramm (1954) stated that sap from *Nicotiana* plants infected with TMV is more strongly inactivating for the virus than sap from healthy plants. The inhibitor is heat stable and soluble in butanol and, therefore, is unlikely to contain protein.

To summarize, although inhibitors of virus infection of varying chemical types appear to be fairly widespread in higher plants there is no good evidence that they play any part *in vivo* in protecting hosts from infection, or in limiting virus increase within an infected plant. They appear to have no effect when viruses are transmitted by insects—the most common method of spread in nature, and their effects on mechanically transmitted virus are usually limited to hosts other than the species from which the inhibitor came. In the intact plant the inhibitory substances presumably do not get into effective contact with the virus, or they may be formed only on rupture of the cells.

XIII. DISCUSSION

Studies on virus inactivation have been of little use so far in the practical control of virus diseases. With certain viruses, such as TMV and potato virus X, which are easily transmitted by mechanical means, inactivation *in vitro* by acids, bases, detergents, or substances such as formaldehyde are used to limit contamination of glasshouses, tools, etc. Inactivation of virus by chemical means within the plant has not yet achieved practical use. There are several situations where such control would be most useful. In field crops subject to severe virus infection, a chemical means of protection would be of great value. For example, sugar beet is regularly infected by the serious yellows disease. Much of the loss of yield due to this virus occurs late in the growing season. A spray treatment which delayed disease development for only a few weeks would be useful. Stocks of many economically important plants are completely infected with viruses. Chemical treatment to free individual plants of the viruses would enable nucleus stocks of virus-free plants to be built up. In such treatments severe but temporary damage to the host plant would not be important. There are several claims for such cures but none of these appears to have been adequately confirmed. For freeing plants from virus infection heat treatment at present offers much better prospects of success and is already in use both to establish virus-free lines of particular varieties of a crop (e.g., strawberries) or as a routine measure for vegetatively propagated material to be planted in the field (e.g., sugar cane).

Recent work has demonstrated the vital role of RNA for the infectivity of viruses. In the following summary of the effects of various inactivating agents it is assumed that most operate directly or indirectly on the RNA of the virus.

1. *In vitro inactivation*

a. *Direct effects on the RNA*

(i) Radiations such as X-rays and ultraviolet light appear to act directly on the RNA within the intact virus particle. It is likely that inactivation by X-rays involves rupture of the polynucleotide chains, and that one such break within a particle is sufficient to inactivate it.

(ii) Heating under appropriate conditions may inactivate certain "spherical" viruses such as tomato bushy stunt without any detectable alteration to the protein part of the virus, and as far as is known without release of RNA from the virus. Presumably such treatment causes some disorganization of the RNA within the virus particle. With the rod-shaped viruses, heat inactivation is more closely associated with disorganization of the virus protein.

(iii) Some chemical reagents (e.g., formaldehyde) may inactivate intact virus particles by combining directly with certain vital groups in the RNA.

b. *Indirect effects on the RNA.* It seems very likely that a major function of the virus protein is to protect the RNA. Many inactivating agents such as heat, mild alkali, or enzymes cause destruction of the protein or remove it from the RNA. Such removal of protein in solution at normal temperatures would lead to rapid thermal inactivation even if the treatment in question had no direct effect on the RNA. For example, the protein of potato virus X is hydrolyzed by certain proteolytic enzymes. Such enzymes presumably have no direct effect on the RNA (unless the preparation is contaminated with nuclease activity) but the RNA would be rapidly inactivated in solution at room temperatures once the protecting protein is removed.

c. *Substances combining with the virus.* Many inhibitory substances can combine with virus *in vitro* without causing any irreversible change in the virus. It has often been suggested that such combination is involved in the inhibition but there is no good evidence in most instances. The combination of specific antibody protein with the virus is perhaps the only example where it is at present reasonable to assume that the combination is essential for the inhibition. It has been suggested that the antibody protein prevents attachment of the virus to some specific receptors in the cell. There is, as yet, no evidence for the existence of such receptors for plant viruses, and an alternative possibility is that the antibody prevents the release of the RNA from the virus protein at an early stage of infection. If either of these ideas is correct, the virus particle is presumably not destroyed on inoculation to a leaf and could be recovered, at least with some viruses such as TMV.

2. *In vivo inactivation*

a. *Inhibition of virus establishment*

(i) Killing of wounded cells: It has been suggested that some inhibitors such as those in certain extracts from plants may kill cells which are wounded at the time of inoculation and thus prevent virus establishment. This possibility has not yet been demonstrated for any treatment.

(ii) Sequestration of some factor essential for virus establishment: With the T5 bacterial virus calcium ions are necessary for the release of the nucleic acid into the host cell. Experiments with organic acids and metal ions suggest that certain plant viruses may also require the presence of metal ions for successful establishment, and that certain substances may prevent infection by the sequestration of such ions.

(iii) An early stage in virus establishment probably involves release of the RNA from the protein of the invading particle. The inhibition of virus establishment by ribonuclease may well involve attack by the enzyme on the naked RNA at a stage before virus multiplication has begun.

b. *Inhibition of virus increase.* With bacterial viruses where a single cycle of virus multiplication can be studied it has frequently been possible to relate the inhibitory effects of a treatment to some definite step in virus multiplication. With plant viruses, however, very little is known either about the way the virus multiplies or about the mechanism by which inhibitors interfere with the process. Some treatments may block the formation or utilization of components essential for virus synthesis but there is no definite evidence that such interference does occur. The only change in the virus known to accompany an inhibition of virus increase is the incorporation of purine and pyrimidine analogues into the virus RNA. Such analogues could inhibit virus multiplication through the formation of nonfunctional RNA, but this may not be the only way in which these compounds act.

c. *Inactivation of existing virus.* Holding plants at high temperatures may lead to inactivation and destruction of existing virus but the mechanism by which this takes place is unknown. It almost certainly involves changes in the RNA within the virus particles and perhaps escape of the RNA from the protein with its subsequent destruction by plant nucleases. Inactivation of virus *in vivo* by radiations such as ultraviolet light probably involves mechanisms very similar to those causing inactivation *in vitro*.

As with many other aspects of plant virus research, further investigation of inhibitory effects on virus establishment and multiplication would be greatly facilitated by the development of a system where all or most cells could be infected simultaneously, and a single cycle of virus multiplication studied.

In experiments on virus inactivation there are many pitfalls for the unwary, ranging from proteolytic enzyme preparations which contain nucleases to green dyes which mask disease symptoms. Nevertheless, such investigations have given us much useful information about the structure and biological activity of viruses, and should prove even more fruitful in the future, since the vital role of the virus nucleic acid is now widely appreciated.

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CHAPTER 13

Physiology of Fungitoxicity

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I. INTRODUCTION

Two matters appear to be of prime importance in the physiology of fungitoxicity. The first concerns the ways that fungus cells are affected by fungicides and the second involves the factors responsible for selective action of fungitoxic compounds. The two are related and must be discussed together because the same kinds of cellular structures and physiological processes are often involved in both. It will be the object of this chapter to examine the morphological features and vital activities of fungal cells as they are related to fungitoxicity and specificity. The discussion will not be confined to plant pathogens and common fungicides but will include other organisms and other toxicants whenever they provide more appropriate illustrations of the points to be made.

Fungicidal physiology begins when a fungus cell and a toxicant are associated in the same environment. Theoretically, at least, a toxicant need not act directly on the fungus cell in order to affect its growth and development. Removal or alteration of a metabolite in the environment or inactivation of an extracellular enzyme would be sufficient in certain instances to prevent growth or to render an organism ineffective as a plant pathogen. However, in the case of the fungicides presently used in plant disease control, direct action on the cell almost surely accounts for their toxicity. The fact that protective fungicides must act on spores that generally contain an internal food supply sufficient to carry them through the primary stages of infection of the host emphasizes the operation of direct rather than indirect effects. Furthermore, most fungicides developed in recent years have been discovered in screening procedures involving germination tests in which dependence on extracellular substances, except water, is at a minimum. If we accept as fact that the majority of fungicides must come in direct contact with the cells they affect and that in most instances they must enter these cells, we should first consider how fungicides, generally characterized by low solubility in water, are mobilized and how they negotiate the possible barriers that may separate them from their ultimate sites of action.

II. MOBILIZATION

Several theories have been advanced to explain how deposits of relatively insoluble toxicants on leaf surfaces or glass slides are made available in quantities sufficient to kill fungus spores. Among the agents and mechanisms that have been proposed as being involved in mobilization are atmospheric moisture, plant excretions, spore secretions, and ability of spores to accumulate toxicants from very dilute solutions.

On the subject of mobilization, which has been covered fully by Horsfall (1956), suffice it to say that Bordeaux mixture and other insoluble copper toxicants have been the subjects of the major portion of the early investigations in this area. Most of the early workers favored CO_2 and ammonia dissolved from the atmosphere in rain water and dew as the solubilizing agents for Bordeaux mixture (McCallan, 1949). Curtis (1944) proposed that guttational water may play a role in increasing phytotoxicity of insoluble copper fungicides and showed that water of guttation when added to deposits on glass slides increased the fungitoxicity of Bordeaux mixture and of other copper fungicides presumably by increasing their solubility. Guttational or meteoric water at most must play only a supplemental role in mobilization of Bordeaux deposits because on glass slides in the laboratory dried deposits of Bordeaux mixture are toxic to spores applied in drops of distilled water (McCallan, 1949).

On glass slides, and possibly also on foliage, secretions from spores may be important in mobilization of insoluble copper fungicides. McCallan and Wilcoxon (1936) showed that secretions from fungus spores increase the toxicity of Bordeaux deposits and that those spores most active in secretion were most sensitive to this fungicide. Malic acid as well as amino acids in the secretions from conidia of *Neurospora sitophila* are apparently responsible for an increase in toxicity. Wain and Wilkinson (1943, 1946) also found that filtrates from suspensions of spores of *N. sitophila* are active in solubilizing and increasing the toxicity of deposits of Bordeaux mixture. Horsfall *et al.* (1937) and Marten and Leach (1944) showed that toxicity of copper oxides is related to their solubility in aqueous glycine solutions.

While many fungitoxicants are only very slightly soluble in water, the amount of toxicant entering water from residues without the aid of solubilizing agents is probably more than sufficient to prevent germination of sensitive spores, especially when the concentration of spores is low. With less sensitive fungi or with high concentrations of spores, however, the rate of solubilization may be a factor in limiting effectiveness. The ability of spores to accumulate large quantities of toxicants from dilute solutions is probably by far the most important factor in explaining the effectiveness of toxicants of low solubility. It has been recognized for some years, for example, that conidia of *Plasmopara viticola* (Delage, 1932) and of *Monilinia fructicola* (Marsh, 1945) can accumulate copper to a concentration much greater than that present in the ambient solution. It is now recognized that limited solubility in water is an essential characteristic of many types of effective fungicides.

III. UPTAKE

With the advent of radioisotope tracer techniques, the problem of studying the accumulation of toxicants by fungus cells was facilitated and these techniques have been used in extensive studies of the uptake of fungitoxicants at the Boyce Thompson Institute for Plant Research (Miller and McCallan, 1955). Among the toxicants studied were dichlone (2,3-dichloro-1,4-napthoquinone), 2-heptadecyl-2-imidazoline, and ions of silver, mercury, cadmium, zinc, and cerium. It was shown that the major portion of a toxicant ultimately taken up by a spore is removed from solution during the first 30 seconds of exposure and that affinity of conidia for these toxicants is very great (Miller *et al.*, 1953b). 2-Heptadecyl-2-imidazoline, for example, is accumulated in some instances to the extent that the concentration associated with the spores is 10,000 or more times greater than the original concentration in the solution in which the spores were suspended. Once these toxicants are taken up they are not readily removed by washing the spores with distilled water (Miller *et al.*, 1954). The response of the spores is not dependent solely on the concentration of toxicant in solution, but on the amount of toxicant per spore, a factor which has not been fully appreciated by some investigators. Considerable quantities of these toxicants are required to inhibit germination by 50%. Compounds such as 2-heptadecyl-2-imidazoline with a low innate toxicity probably owe their usefulness mainly to their ability to accumulate in large quantities in the biophase.

Large quantities of cationic toxicants such as copper and silver are also taken up from dilute solutions by dormant ascospores of *N. tetrasperma* (Lowry *et al.*, 1957). The evidence indicates that these toxicants are adsorbed at the surface of the dormant ascospores and they do not penetrate to susceptible sites until the spores begin to germinate. In the case of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine and derivatives, Burchfield and Storrs (1957) have shown that about 1500 μg. of toxicant per gram of conidia of *N. sitophila* must be accumulated to produce 50% inhibition of germination.

The uptake of members of an homologous series of *N*-*n*-alkylethylenethioureas by yeast cells and by conidia of *M. fructicola* was investigated by Ross and Ludwig (1957). Toxicity in this series is related to physical rather than to chemical properties and the differential toxicity of the various members of the series is explained simply by the nature of their partitioning between the biophase and the ambient solution. As the length of the alkyl side chain is increased up to and including the octyl homologue, the proportion of toxicant taken up by the cells increases and so does toxicity. The aqueous solubility of ethylenethiourea is so high that it is not partitioned to any appreciable extent into the biophase and thus it is ineffective as a toxicant.

It is apparent that many fungicides in current use must be accumulated by fungal cells in relatively large quantities before their toxic effects are evident. On a microgram-per-gram basis, they are much less effective than penicillin, certain insecticides, and other biocidal agents (McCallan, 1957).

One of the primary problems related to the uptake of toxicants is that of distinguishing between adsorption at the surface of the cell and entrance into the cell. Some aspects of this problem are discussed in a later section.

IV. SURFACE OF THE CELL IN RELATION TO TOXICITY

The cell wall and possibly the outer surface of the cytoplasmic membrane are vital areas in direct contact with the external environment. These structures may represent sites of toxic action. Compounds that act at these sites do not need to negotiate differentially or semipermeable membranes and thus may differ in their physical properties from those that act within the cell. However, the principal significance of the cell wall and cytoplasmic membrane for those toxicants that act inside the cell may be that they offer resistance to penetration.

A. *The Cell Wall*

The cell walls of fungi are complex and variable structures. Among the substances reported to make up the cell walls of fungi are chitin, cellulose, glucan (yeast cellulose), pectin, callose, mannans, lignin, proteins, and lipids (Foster, 1949; Kreger, 1954; Falcone and Nickerson, 1956). Although all of these materials are not necessarily present in the wall of any particular fungus, lipids, protein, and one or more of the skeletal compounds, such as chitin, are generally present. Chitin is especially characteristic of the cell walls of the filamentous fungi. The materials comprising the cell walls are probably intimately associated and mutually protected from the effects of external enzymes and other agents. X-ray evidence indicates that, as a rule, the materials are linked by easily hydrolyzable bonds to form complexes (Kreger, 1954). Thomas (1928) found that certain components of the walls of *Fusarium* cells protect other components from the action of chemical reagents.

1. *Interference with Structure and Synthesis of the Cell Wall*

Disruption of cell wall structure, interference with the process of cell wall extension during growth or interference with synthesis of cell walls represent possible mechanisms of fungicidal action that have received relatively little attention in the past. However, the recent demonstration that penicillin can induce the formation of cell-wall-free protoplasts of *Escherichia coli* should emphasize the possible involvement of the cell wall in fungitoxic action. Lederberg (1957) proposed that the bactericidal effect of penicillin in ordinary media may be explained sufficiently on the basis of induced osmotic fragility, probably resulting from the failure of growing cells to form new walls. Results of Goksøyr's (1955) work also point toward interference with synthesis of walls as a possible basis of toxicity of dithiocarbamyl compounds to baker's yeast.

Brian (1949) suggested that griseofulvin affects either directly or indirectly the cell wall or the process of extension of the wall. This antibiotic characteristically causes swelling and various types of distortion of the growing tips of fungal hyphae. He made the interesting observation that among the many species of fungi that he tested (representative of all of the orders), only those reported to have chitin walls are affected. It is entirely possible that other fungitoxicants which cause bursting of fungal cells or swelling of germ tubes and hyphal tips may do so by interference with cell wall synthesis.

There is some evidence that emergence of a germ tube from some fungal spores is preceded by a localized plasticizing of the cell wall through some metabolic process. It has been noted in this laboratory that conidia of *Fusarium roseum* in shake culture become sticky and tend to clump together just prior to germination, indicating that some alteration of the cell wall has taken place. Falcone and Nickerson (1956) have isolated a mannan-protein complex from the cell walls of *Candida albicans*. The protein in this complex contains S-S linkages that can be reduced by a "cell division" enzyme. It was suggested that localized enzymatic reduction of the protein disulfide converts a portion of the "vulcanized," cross-linked wall fabric into a form capable of plastic deformation through which a bud can emerge (Nickerson and Falcone, 1956). A similar change may be necessary for the emergence of germ tubes from certain conidia. Toxicants that react with constituents of the walls of spores, or inhibit enzymes which alter the walls, may prohibit the emergence of germ tubes.

Synthesis of certain components of the cell walls of fungi and the processes that control the physical properties of the walls are probably distinct from those that occur in higher plants. Chitin, for instance, seems to be the main skeletal material in the cell walls of most filamentous fungi and its synthesis involves metabolism that might be selectively inhibited in fungi without injury to the higher plant. The chitin polymer is probably formed at the surface and the enzymes involved would likely be readily accessible to toxicants. Interfering with synthesis of cell walls of plant pathogens would apear to offer greater promise as a means of plant disease control than would an approach directed toward damaging the existing cell wall structure. Analogues of glucosamine might prove to be useful in the selective inhibition of plant pathogens.

2. *Permeation*

The classical point of view has been that the cell wall plays a relatively insignificant role in limiting penetration of compounds of low molecular weight and there is evidence that this may often be the case.

Permeability of cell-wall-free protoplasts of bacteria to low molecular weight compounds, such as sucrose, urethane, and phosphate, is reported to be essentially the same as for intact cells (Weibull, 1956). Owens and Miller (1957) found insignificant quantities of 2-heptadecyl-2-imidazoline, dichlone, and Ag, Zn, Cd, Hg, and Ce ions in the cell wall fractions of spores of *N. sitophila* that had been treated with these toxicants prior to disintegration.

On the other hand, the cell wall may prevent certain substances from reaching the protoplasts. The degree of protection afforded by the walls undoubtedly varies greatly with the species of fungus. Conidia of *Erysiphe polygoni* contain large amounts of water which they do not lose readily even in a dry atmosphere. They also contain large quantities of lipids and Yarwood (1950) has suggested that fatty materials deposited in the cell walls may limit their permeability to water and toxicants such as copper sulfate. Conidia of this fungus will germinate on the surface of 10% copper sulfate solutions (Yarwood, 1945), although copper sulfate is toxic to the hyphae. Such substances as gelatin, peptone, inulin, and divalent succinate ions reportedly do not permeate the outer regions of cells (cell wall) of baker's yeast (Conway and Downey, 1950).

Evidence that the cell walls of higher plants may protect their protoplasts from certain fungitoxicants is provided by the work of Ross and Ludwig (1957). They found in an homologous series of *N*-*n*-alkylethylenethioureas that maximum phytotoxicity falls near the amyl homologue when structures such as seed coats, cell walls, and middle lamellae are present, but when these barriers were removed, the peak of phytotoxicity occurred near the octyl homologue. Uptake of these compounds by discs of potato tuber and by pure cellulose (filter paper) increased in a geometric manner as the length of the alkyl side chain increased up to and including the octyl homologue. These data indicate adsorption of the compounds by cellulose and the authors suggested that adsorption of the higher homologues by the cell walls, seed coats, and middle lamellae probably limits their phytotoxicity. Uptake by yeast cells increased in an arithmetical progression as the length of the alkyl side chain was increased and, as with the exposed protoplasts, maximum toxicity was reached at the octyl homologue. Apparently the walls of baker's yeast, composed primarily of glucan, mannan, proteins, lipids, and a small amount of chitin (Roelofsen and Hoette, 1951), do not protect their protoplasts from the higher homologues of the series.

The protection that the cell wall provides against high molecular weight substances, such as enzymes, is probably of more significance than protection that it affords from toxicants that are of comparatively low molecular weights. Bacterial protoplasts, for instance, are readily attacked by lipase, trypsin, and deoxyribonuclease, enzymes to which the intact bacterial cells are quite insensitive (Spiegelman, 1957).

B. *The Cytoplasmic Membrane*

The structure of the cytoplasmic membrane, its significance as a permeation barrier, and its metabolic functions still remain obscure despite considerable research and speculation. It is generally assumed from indirect evidence that the cytoplasmic membrane is lipoprotein in nature. A generalized pattern of the membrane, consisting of a layer of lipids two molecules thick with a monomolecular layer of protein on the inner and outer surfaces, has been proposed by Davson and Danielli (1943). The cytoplasmic membrane must be considered in fungitoxicity because it may act as a permeation barrier to toxicants or the toxicants may act at this site to inhibit enzymatic activity, to block entry of metabolites into the cell, to alter permeability in such a manner as to cause the loss of cellular constituents, or to cause an irreversible physical disruption of the membrane structure.

1. *Permeation*

It is generally recognized that lipid solubility, or some property closely correlated with it, is important in determining how readily a compound permeates a cell, but that permeation is also influenced in some way by the molecular size of the permeants (Collander, 1957). Inability to permeate the cytoplasmic membrane is sometimes invoked as a possible explanation for the failure of otherwise likely compounds to function as fungicides.

Among the principal reasons for assuming that the cytoplasmic membrane is involved in regulating the entrance of toxicants into the cells are the correlations between toxicity and the proper degree of lipid solubility of compounds and the fact that neutral molecules of many toxicants are frequently more effective than the corresponding ionized forms.

In toxicants exerting their effect through chemical mechanisms, i.e., structurally specific compounds possessing one or more active chemical groups that combine with specific cell constituents, the proper degree of lipid solubility is thought to be a physical property required for permeation to the susceptible sites within the cell and is not involved in their ultimate activity. Thus, when increased toxicity of this type of compound is achieved by the addition of inert lipophilic substituents to the molecule, it apparently results from more ready permeation. This is reported to be the case with the nitrosopyrazoles, in which the nitroso is regarded as the active group (Rich and Horsfall, 1952). Addition of inert hydrophobic substituents to the pyrazole ring increased the oil-water partition coefficient as well as toxicity, and the increased toxicity in this case was attributed solely to the increased ability of the compounds to permeate the cell membrane. This appears to be the case with a number of organic fungicides. With the 8-quinolinols, for example, lipid solubility as well as ability to chelate is required for activity (Block, 1955). It has been postulated that the $-SCCl_3$ group in the captan molecule functions in permeation (Horsfall, 1956), but there is now evidence that this group may be the toxiphore (Lukens and Sisler, 1958).

A somewhat different relationship between permeation and lipid solubility seems to characterize the chemically inert, structurally nonspecific toxicants that act by physical, rather than by chemical, mechanisms. These are compounds that do not owe their toxicity to a specific chemical group but rather to their ability to accumulate in the biophase. The *N-n*-alkylethylenethioureas are representative of this kind of toxicant (Ross and Ludwig, 1957). The relative effectiveness of such substances is related to their partition coefficients between fat-like substances and water. Structurally nonspecific compounds of diverse types have approximately the same potency when they are present in the same proportional saturation in the medium in which they are applied even though their molar concentrations in that medium may differ widely (Ferguson, 1939). This is known as Ferguson's Principle and it seems to apply to all compounds which owe their activity to certain physical-chemical properties that favor their accumulation in the biophase (Albert, 1951). An increase in activity of such compounds as the oil-water partition coefficient increases does not necessarily mean that their ability to permeate membranes was the limiting factor in toxicity. Johnson *et al.* (1954) point out that the same type of bond is formed when such substances dissolve in oil as when they combine with hydrophobic groups of enzymes or other proteins. The correlation between lipid solubility and potency also applies to purified enzymes *in vitro* in the absence of lipids and cellular structure which suggests that hydrophobic portions of proteins may be the sites of action. These sites may occur at the cell surface. However, in the intact cell, lipid barriers at the membrane may have to be negotiated before susceptible proteins are encountered and thus partition between lipid and water may be a measure of their ability to penetrate membranes as well as a measure of their ability to combine with hydrophobic groups in protein molecules. Since water-lipid solubility, surface activity, and adsorbability are somewhat parallel phenomena, one wonders whether ease of penetration of the cell membrane is the only significance of the proper degree of lipid solubility even in the case of structurally specific toxicants.

The biological activity of the ions of weak acids and bases is frequently lower than that of the corresponding undissociated molecules. The differences in activity usually have been attributed to difficulties in permeation by the ionic forms. Permeation is affected by the greater relative size of ions, due to hydration, by their charge resulting either in repulsion or fixation by adsorption, and to their generally lower solubility in lipids. Simon and Beevers (1952) report that pH changes in the range 3 to 6 have no effect on the concentration of iodoacetic acid ($pK = 3.1$) required to produce 50% inhibition in the fermentation of glucose by zymase, but the concentration required to halve the fermentation rate of intact yeast cells was thirteen times greater at pH 6.0, where the compound existed almost entirely in the ionic form, than at pH 3.0 where the concentrations of ions and neutral molecules were approximately equal. This evidence suggests that the cytoplasmic membrane restricts permeation of the ionic form of this toxicant. On the other hand, it is the ionic forms rather than the neutral molecules that are responsible for toxicity of some compounds. This is the case for the acridine antibacterials (Albert, 1951). It is probable, however, that they act at the surface of the cells rather than internally, because the addition of aliphatic side chains to the molecule to aid permeation reduces their antibacterial action.

It might be pointed out here that mitochondrial and nuclear membranes may also constitute permeation barriers that may be of considerable significance in fungitoxicity. Both mitochondria and microsomes are rich in lipids and there is evidence that the membranes of mitochondria are semipermeable (Lindberg and Ernster, 1954).

There is considerable variance of opinion on the significance of the cytoplasmic membrane as a permeation barrier to normal metabolites and to toxicants. Mitchell and Moyle (1956) hold that the cytoplasmic membrane of *E. coli* is relatively impermeable even to phosphates and sodium chloride. Conway and Downey (1950) concluded that arabinose, galactose, ions of Na, Cl, and K, and many other substances enter the outer region of baker's yeast, but are apparently barred by the cytoplasmic membrane or enter only very slowly into the cell. Mandels (1953a) achieved selective destruction of surface-located carbohydrases in spores of *Aspergillus luchuensis* and *Myrothecium verrucaria* with 0.1 *N*. HCl without significantly affecting other metabolic processes or the subsequent rate of growth. It is difficult to conceive of such selective action of hydrogen ions without the participation of a membrane that excludes most of them from the cytoplasm. Conidia of *Erysiphe polygoni* germinate well on a 10% copper sulfate solution (Yarwood, 1945) and one must conclude as Yarwood (1950) did, that this resistance is best explained as a failure of the toxicant to permeate into the spores. Lipids in the cells wall, however, may be more important than the cytoplasmic membrane in excluding the toxicant in this case.

On the other hand, there is evidence that the cytoplasmic membrane in certain fungi and other organisms may not constitute a very significant barrier to the permeation of metabolites and toxicants of low molecular weight. Some plant physiologists (cf. Epstein, 1956; Robertson, 1957) have experimental evidence to indicate that a cytoplasmic membrane may not impede the permeation of ions into the cytoplasm of cells of roots of higher plants. Similarly, Roberts *et al.* (1955) concluded from their experiments with *Escherichia coli* that permeation of many compounds of low molecular weight into most or all of the water space of the cell proceeds unimpeded by a membrane at the surface. Inorganic ions, phosphorylated sugars, amino acids, and glutathione are reported to permeate readily. Less extensive experiments with the yeast, *Torulopsis utilis,* indicated that there is no lack of permeability in this organism. It is well known that intact cells of many organisms do not metabolize the phosphate esters of sugars, such as fructose-1,6-diphosphate, when they are supplied externally. However, these compounds can be isolated from living cells and they are readily metabolized by cell homogenates. It would appear that in most cases the failure of intact cells to metabolize phosphorylated sugars must be one of permeability, but, according to Roberts, *et al.* (1955), some other reason must explain the failure of *E. coli* to metabolize fructose-1,6-diphosphate.

Although the experiments of Miller *et al.* (1953b, c, 1954), concerning the uptake of toxicants by fungus spores, were not designed specifically to show whether the toxicants actually enter the cytoplasm, ready permeation was inferred. In this connection, McCallan (1957) indicated that permeation of the fungus spore may not be the obstacle it was once considered to be. In further studies of uptake, Owens and Miller (1957) attempted to determine whether the toxicants, dichlone, glyodin base (2-heptadecyl-2-imidazoline), and ions of five heavy metals, actually enter the cytoplasm of conidia of *N. sitophila* and of *A. niger.* When conidia were exposed to sublethal doses of the fungitoxicants and then disintegrated by sonic treatment, essentially all of the toxicant that was taken up from the medium was found in the supernatant fraction and in the fraction containing particulates corresponding in size to the mitochondria and microsomes. Even though the spore walls of *Neurospora* account for 35% of the weight of the conidia, the fraction containing cell wall fragments retained only insignificant amounts of any of the toxicants.

Experiments with uptake and permeation have dealt largely with successful toxicants and fungi sensitive to them and they indicate that the toxicants are capable of entering and exerting their effect in the cytoplasm of cells. Experiments of this type designed specifically to compare uptake of a toxicant by sensitive and insensitive or immune organisms, should indicate to what extent differential uptake and permeation are responsible for selective toxicity. This is an area of investigation that has as yet received little consideration.

One might conceive of the following differences between resistant and sensitive species or between pathogen and host that would account for their differential uptake and response to a toxicant: (1) the toxicant does not permeate into the cytoplasm of either the resistant or susceptible species, but acts at sites on the surface of sensitive cells. Resistant species do not possess such sensitive sites at this locus; (2) the toxicant permeates readily into both resistant and susceptible cells, but only the latter possess highly sensitive sites internally; and (3) both resistant and susceptible species possess equally susceptible sites internally, but differentiation permeability to the toxicant at the cytoplasmic membrane or at other levels in the cell accounts for selective toxicity. It should be recognized, of course, that within these categories the differences need not be absolute, but may be relative and that various combinations may occur.

2. *Interference with Activities at the Cytoplasmic Surface*

Species may differ more widely in their surface structure and activity than they do internally. Metabolic activities occur at the surfaces of cells. Digestion of extracellular, nonutilizable substrates into forms that may be absorbed by the cell, active transport, self-maintenance, and self-replication are biochemical functions attributable to the cell surface (Rothstein, 1954). Among the enzymes reported to be located at the surface of fungus cells are certain carbohydrases (Wilkes and Palmer, 1932; Mandels, 1953a), phosphatases (Rothstein, 1954), and ascorbic acid oxidase (Mandels, 1953b). It has been suggested that the protein of the cell membrane functions not only by endowing the membrane with certain structural properties, but that it also has enzymatic properties (Mandels, 1953a). Both extracellular enzymes and cell wall material must be synthesized at the surface of the cell or else some mechanism must be assumed that will allow passage of these substances of high molecular weight through the plasma membrane.

It is not known how frequently toxicity is a result of interference with vital activity at the cell surface, because it is usually difficult to distinguish between interference at this site and that occurring internally. There is evidence, however, that some toxicants may act primarily by interfering with activity at this locus. For instance, the predominant, acute, toxic action of uranium to yeast is reported to involve combination with multiphosphate compounds at the cell surface. The uptake of fermentable sugars is blocked, but metabolism of other substrates remains essentially unaffected. Endogenous respiration and oxidation of acetate, pyruvate, lactate, and alcohol are insensitive to a concentration of this inhibitor that blocks sugar uptake (Rothstein, 1954).

In *N. sitophila,* uranium has an effect similar to that noted in yeast (Cochrane *et al.*, 1957). Concentrations of uranyl nitrite that completely inhibit respiration of glucose do not affect the oxidation of acetate, ethanol, or glycerol. The conclusion was drawn that uranium must block reactions near the cell surface which are essential for glucose transport.

Goksøyr (1955) concluded that certain dialkyl dithiocarbamyl compounds inhibit the growth of *Saccharomyces cerevisiae* by interfering with thiol-activated enzymes located at the cell surface. His data show that the only decrease in metabolic activity (when related to degree of growth) that is brought about by these dithiocarbamates is to be found in processes leading to synthesis of cell wall material which, according to Rothstein (1954), most probably occurs at the surface of the cell which is in direct contact with the cell wall.

Selenate competes with sulfate for absorption sites on the cell membrane of algae (Shrift, 1954a). Selenate can be largely excluded from algal cells if sufficiently high concentrations of sulfate are present. Growth of histidine-requiring mutants of *N. crassa* is inhibited when certain combinations of amino acids are provided together with histidine (Mathieson and Catcheside, 1955). Growth of the wild type of this species is not affected by such treatment, nor is growth of the histidine-requiring mutants if they are allowed to take up histidine prior to the addition of the inhibiting amino acid combinations. It was shown that this inhibitory combination of amino acids prevents uptake of histidine by both the wild type and the histidine mutants. The former are able to synthesize the histidine they need while the latter are not. This seems to be an excellent illustration of toxicity based on interference with the entrance of a metabolite into the metabolic system. A similar mechanism of interference may explain why certain toxicants that are analogues of metabolites of both host and pathogen are toxic only to the pathogen. Woolley (1952) discussed instances wherein an antimetabolite is toxic only when the organism requires the corresponding metabolite from an external source. Since many fungi are apparently capable of invading the host plant in the absence of exogenous nutrients, spraying plants with compounds that interfere with uptake of metabolites would not appear to offer promise as a means of plant protection. On the other hand, cells of higher plants are able to synthesize organic metabolites that many pathogenic fungi must obtain from their environment. It is apparent, therefore, that toxicants which block the entrance of such metabolites into fungal cells might be useful chemotherapeutants.

3. *Effects on Membrane Permeability and Structure*

Some toxicants may act by disrupting the cytoplasmic membrane or by altering its permeability so that cellular constituents are lost into the surrounding medium. At lethal concentrations, silver causes conidia to lose considerable quantities of phosphorus to the ambient solution. Interference with permeability represents an important characteristic of the action of silver (Miller and McCallan, 1957a). Tyrocidine causes bacterial cells to leak their soluble constituents, presumably by alteration of the cellular membrane (Hotchkiss, 1944). A number of cationic and anionic surface-active agents produce the same effect as tyrocidine (Hotchkiss, 1946) and their toxicity is attributed to an irreversible damage to the cytoplasmic membrane. According to Hotchkiss, ionic surface-active agents combine with oppositely charged ions at the surface of the cell and if the hydrophobic portion of the toxicant also has proper affinity for the surface, irreversible damage to the cell membrane results at very low concentrations. Soluble constituents are released from the cells and this is followed by autolysis with further release of cellular constituents. It would not be surprising if both glyodin and *n*-dodecylguanidine acetate (Cyprex) were reported to cause leakage of metabolites from fungal cells, because both are cationic, surface-active agents. Newton (1953) and Few and Schulman (1953) showed that polymyxin induces loss of cellular constituents from bacterial cells and suggested that it combines with components of the cell surface, thus altering permeability of the cells. Algal cells lose cellular constituents when treated with polymyxin (Galloway, 1958). Lowry and Sussman (1956) showed that polymyxin also causes loss of cellular constituents from ascospores of *N. tetrasperma* after they begin to germinate and are susceptible to osmotic damage. It is not absolutely certain, however, that changes in permeability may not be a secondary effect of intracellular origin. Polymyxin also prevents uptake of methylene blue, presumably by blocking sites at the surface of the ascospores.

The significance of loss of soluble cellular components in fungicidal action is not entirely clear, because spores of some fungi can apparently lose considerable quantities of certain constituents when suspended in distilled water and they may lose additional quantities when treated with sublethal doses of certain fungitoxicants without any impairment of their ability to germinate (Miller and McCallan, 1957b). Spores of

N. sitophila, for example, when treated with an *s*-triazine lose cellular constituents such as phosphorus, and this loss is attributed to changes in permeability induced by the toxicants, but the spores germinate well and it appears unlikely that effects on permeability alone are primarily responsible for toxicity of these fungicides (Burchfield and Storrs, 1957).

V. Damage to Physical Structure and Organization

In addition to plasma membranes there are other structures and a great deal of organization that must be maintained in a living cell. Irreversible disruption of structure or organization results in malfunctioning and death of cells. Kauzmann (1954) presents a discussion of the types of weak, secondary, intramolecular bonds responsible for maintaining the structural patterns of enzyme proteins. These include hydrogen bonds, ionic bonds, van der Waals' forces, cystine disulfide bonds, and "hydrophobic bonds." The last type involves the adherence of hydrophobic amino acids residues of proteins to each other as a result of their tendency to avoid the aqueous phase. Hydrophobic bonds are considered to be particularly important in maintaining structural stability of protein molecules. Rupturing of the bonds responsible for maintaining protein structure leads to denaturation. Undoubtedly, linkages of these several types are also of major significance in joining protein molecules in the cytoplasm, in linking proteins and lipids in the cell membrane, and in linking nucleic acids with proteins in the chromosomes, mitochondria, and microsomes. Changes in viscosity of protoplasm are probably associated with the formation and breaking of these linkages. Toxicants that rupture the linkages responsible for maintaining stability of cell structure may cause irreversible physical damage and loss of organization in the cell.

Since the toxicants which act by physical mechanism have much greater affinity for lipid-dissolving materials than for water, they probably collect at hydrophobic junctions and weaken or disrupt these linkages, causing damage to physical structures. It has been suggested that organic solvents and detergents denature proteins in this manner (Kauzmann, 1954). Dodecylsulfate is commonly used to dissociate nucleic acid from protein in viruses. Its action is probably that of disrupting ionic and other linkages joining the proteins and nucleic acids. 2-Heptadecyl-2-imidazoline is known to associate with particulate fractions of the cytoplasm of fungal cells (Owens and Miller, 1957) and, at toxic concentrations, it may disrupt physical structure. On the other hand, these ionic surface-acive toxicants may prevent attachment of enzymes to coenzymes or to substrates at concentrations lower than those necessary to disrupt structural features of the cell. Other aspects of physical toxicity as it affects chromosomes and mitotic behavior are discussed in a later section of this chapter.

VI. Interference with Intracellular Metabolism

While there is evidence that some fungitoxicants may act at the surface of the cell it would seem to be a safe assumption that most toxicants exert their effects internally. The vast number of enzymatic reactions involved in release of energy from substrates and in syntheses of materials in the cytoplasm undoubtly account for the major portion of vital cellular activity with which a toxicant might interfere in a lethal manner.

It is becoming increasingly clear that there is much similarity between the vital intermediate metabolism of fungi and that of other organisms. The vitamins, coenzymes, amino acids, purines, and pyrimidine, as well as the majority of other known metabolites of a vital nature, are identical in fungi and in other organisms. The fatty acids—palmitic, stearic, oleic, and linoleic—which are found in largest quantities in lipids of fungi (Foster, 1949)are also abundant in lipids of higher plants (Bonner, 1950). Likewise, the general metabolic pathways of fungi seem to be very similar to those of other organisms. The operation of the Krebs cycle in the filamentous fungi and in yeasts has been demonstrated by several investigators (Foulkes, 1951; Krebs *et al.*, 1952; Roberts *et al.*, 1955; DeMoss and Swim, 1957; Bonner and Machlis, 1957; Moses, 1957). Pentose cycle reactions have been demonstrated in *Penicillium chrysogenum* by Sih, *et al.* (1957) and cytochrome pigments were found in all of 47 species of fungi investigated by Boulter and Derbyshire (1957). It would seem logical that protein and nucleic acid synthesis would proceed in fungal cells in essentially the same manner as it does in cells of other organisms.

One meets with considerable difficulty in explaining selective toxicity of fungicides on a basis of differences in metabolism of host and pathogen since their general metabolic processes are apparently very similar. Unique vital processes are more characteristic of the higher forms than they are of fungi. Thus, the possibilities of selectively controlling a group of organisms by interfering with activities unique to that group are more promising with the more complex forms of life. However, in different organisms there are undoubtedly many minor variations in metabolic pathways and in enzymes that have the same function. These differences may be of considerable significance in selective toxicity. Lysine biosynthesis, for instance, appears to proceed by a pathway in yeast and in *Neurospora* which is different from that in *E. coli*. ϵ-Amino adipic acid is a precursor for lysine in these fungi, while diamino pimelic acid is a precursor in *E. coli* (Kamin and Handler, 1957). Alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase from yeast differ in certain of their properties from the corresponding enzymes from animal sources (Velick, 1954). The protein moiety of cytochrome c from heart muscle differs from that of cytochrome c from *Ustilago sphaerogena* (Neilands, 1952). Saz *et al.* (1956) proposed that an altered enzyme may explain the resistance of a strain of *E. coli* to chlortetracycline. In cell-free preparations from a sensitive strain of the organism, the organic nitroreductase was inhibited by bacteriostatic concentrations of the antibiotic while in similar preparations from resistant cells the enzyme was likewise resistant to the antibiotic. One of the differences noted was that the enzyme from the resistant strain contained firmly bound, conjugated flavin, while the flavin of the nitroreductase from the sensitive strain was easily dissociable (Saz and Martinez, 1956).

Differences in metabolic pathways and enzymes may be of considerable significance in determining the effectiveness of toxicants that are highly specific in their modes of action. They can, however, hardly account for the selective control of pathogenic fungi that has been attained with toxicants that are not selective in their modes of action, but are capable of reacting with common chemical groups, –SH for example, that may occur at many points in most living cells. In these cases differences in permeability and in a number of other factors are probably of more significance than differences in susceptibility of enzymes. There is little doubt that the heavy metal fungitoxicants and possibly some of the organic fungicides used in the practical control of plant diseases would be about equally destructive to the metabolism of higher plants as of fungi if the concentrations attained in the cytoplasm of both types of cells were equivalent.

Fungitoxicants that affect cells by interference with intracellular metabolism might be considered as falling into two general groups: (A) Those analogues of metabolites that disrupt normal processes by competing with metabolites and, (B) those that affect metabolism by virtue of their ability to interfere with enzymes, coenzymes, metal activators, or substrates in some noncompetitive manner.

A. *Effects of Antimetabolites*

It appears that most of the toxicants that are highly specific in their modes of action are analogues of metabolites. These toxicants, because of their close similarity in structure or characteristic chemical reactivity, to normal metabolites interfere in a reversible manner with the utilization of normal metabolites by substituting for them in metabolic processes. They are often referred to as antimetabolites. Many analogues of metabolites have been reported since Woods (1940) first showed that sulfanilamide is an analogue of the essential metabolite, *p*-aminobenzoic acid.

The primary effect of such toxicants may be limited to a single reaction in some cases, although if the affected reaction is in some primary stage of metabolism, many other reactions will be affected secondarily. For instance, prevention of incorporation of any one amino acid into protein by an effective amino acid analogue prevents utilization of virtually all other amino acids as well (Halvorson and Spiegelman, 1952). On the other hand, much of the primary metabolism of a cell may remain unaffected when an analogue interferes with some more advanced stage of metabolism. Sulfanilamide is an antimetabolite for *p*-aminobenzoic acid in fungi as well as in bacteria (N. S. Dimond, 1941), but it does not affect oxidation of sucrose by cells of *N. crassa* (Tatum and Giese, 1946) or of glucose by conidia of *F. roseum* (Sisler and Marshall, 1957) at concentrations that inhibit growth. There also appears to be no effect on the processes of assimilation in resting cells of *F. roseum,* because the treated cells increase in weight to the same extent as do the untreated cells.

In some instances an analogue may participate in a curious course of events in which it is utilized by a cell in the synthesis of another analogue which is actually responsible for toxicity. Fluoroacetic acid is a case in point. It is an analogue of acetic acid and is reported to be synthesized by the cell into fluorocitric acid which interferes with aconitase and prevents the conversion of citrate to isocitrate (Peters, 1952). It may well be that the fluoroacetate is also incorporated into lipids and other cellular components that are normally built up from acetate. Selenate, an analogue of sulfate, interferes with sulfate utilization by cells of *Chlorella vulgaris* and is also synthesized into selenomethionine, an antimetabolite of methionine (Shrift, 1954b). An analogue of tryptophan, 7-azatryptophan, does not prevent protein synthesis in *E. coli,* but does prevent the development of a number of enzyme activities (Pardee *et al.,* 1956). The proteins that are synthesized in this case are probably inactive analogues of the normal enzymes. These are examples of lethal synthesis, a subject that is discussed in more detail by Markham (1958).

Most of the antimetabolites that have been described, either coincidentally resemble, or have been patterned deliberately after vital metabolites that are common to many species. They are not specific in the sense that they resemble a metabolite peculiar to a particular group of organisms. Horsfall (1956) has compiled a list of compounds that function as antimetabolites in fungi. Practically all of the compounds that he listed, however, are potential antimetabolites for other organisms as well and many of the compounds listed by Woolley (1952) are analogues of metabolites common to numerous species. With our present understanding of comparative biochemistry the chances of deliberately designing and synthesizing an analogue specific for a metabolite peculiar to fungi are not as good as are the chances of designing one specific for higher organisms with more complex and highly specialized activities. However, as our understanding of the details of differences between the metabolism of cells of various organisms increases, the chances of selecting or synthesizing an antimetabolite for a particular process should also increase and offer promise of highly selective control of plant pathogenic organisms.

Remarkably enough, selective inhibition of certain species of organisms without affecting others has often been accomplished with compounds that are apparently analogues of metabolites common to both the sensitive and resistant species. Woolley (1952) has provided an excellent example to illustrate this point. Pyrithiamine is an analogue of thiamine and is known to be toxic to certain higher animals, invertebrates, fungi, and bacteria, but there are many species of fungi, bacteria, and higher plants that are unaffected by this compound. There is little doubt that thiamine is a metabolite in both resistant and susceptible species. Resistant and susceptible forms are in some cases closely related and genetic differences between many of the resistant and susceptible organisms must be very slight. Therefore, selective toxicity of pyrithiamine, based on gross and fundamental morphological and physiological differences, does not seem likely, especially since widely separated species have a similar degree of susceptibility. Woolley (1952) gives a thorough discussion of a number of factors that may be involved in selective toxicity in such cases.

Very few of the well-known fungicides appear to be antimetabolites and even those that have been reported to function in this fashion seem capable also of other, less specific modes of action. Among those for which antimetabolitic activity has been suggested are 2-heptadecyl-2-imidazoline, dichlone, sulfur, and captan. Both guanine and xanthine antagonize the toxicity of 2-heptadecyl-2-imidazoline to *Sclerotinia fructicola* and the antagonism is of the competitive type (West and Wolf, 1955). This would indicate that this toxicant is an antimetabolite for these purines, but it is a cationic surface-active agent and, thus, one might expect it to be capable also of other less specific effects. Germicidal activity is generally characteristic of ionic surface-active agents some of which are known to cause osmotic damage and to disrupt macromolecular structure. McCallan *et al.* (1954) showed that concentrations of 2-heptadecyl-2-imidazoline that reduce oxygen consumption of certain fungal conidia by 50% also reduce their germination by about the same amount. This implies that the fungicide may affect processes other than xanthine and guanine metabolism. Dichlone resembles vitamin K in structure and Woolley (1945) demonstrated that vitamin K will reverse the fungitoxicity of dichlone in a competitive manner, but only over a very limited range of concentrations of the toxicant. It was recognized, however, that the reactive chlorine atoms in the dichlone molecule might well confer on it toxicity that is unrelated to any effect that it may exert as an antimetabolite (Woolley, 1952). McCallan (1957) considered the hypothesis that sulfur may substitute for oxygen in the respiratory systems of fungi and that it might exert its toxic effects by competition with oxygen. Since sulfur actually stimulates oxygen consumption in fungi and since cyanide does not inhibit the capacity of fungi to convert sulfur into H_2S, it was considered unlikely that sulfur acts by replacing oxygen in the respiratory process. There remains the possibility, however, that sulfur may compete with other hydrogen acceptors in cells and thus disrupt their metabolism, as has been suggested by Miller *et al.* (1953a). Hochstein and Cox (1956) demonstrated competition between captan and cocarboxylase in preparations of dried brewers' yeast. It was also shown that in conidia of *F. roseum* that were treated with captan, pyruvic acid accumulated to a greater extent than in untreated conidia as would be expected if decarboxylation of pyruvate were inhibited. Although thiamine pyrophosphate offset the effect of captan in yeast preparations, it did not protect living cells and impermeability of the cells to this coenzyme was considered as a possible explanation of its failure to protect. It was suggested that captan may owe its toxicity to interference with enzymes that require thiamine pyrophosphate.

Xanthine and guanine, oxygen, vitamin K, and thiamine pyrophosphate are metabolites that are by no means peculiar to fungi. If the toxicants, dichlone, sulfur, and captan do behave as antimetabolites in fungi, then there must be differences other than metabolic ones that are at least partially responsible for rendering these compounds more toxic to fungi than to higher plants and animals.

Actually, inhibition of metabolism by competition may be more common than is presently suspected since some toxicants not now regarded as antimetabolites may permeate so readily and have such high affinity for the susceptible enzyme that they would accumulate in cells to such an extent that the range of concentration of normal metabolite required to demonstrate the competitive nature of the inhibition cannot be attained in the intact cell. This would be the case especially when the cell is impermeable to the normal metabolite, i.e., coenzyme supplied externally.

Many cationic and anionic toxicants may act in the cell as inhibitory analogues of essential ions. This would constitute a somewhat more general type of competitive inhibition than that which is commonly associated with antimetabolites. According to MacLeod and Snell (1950), certain metallic ions which inhibit growth of bacteria might be regarded as structural analogues of other metallic ions which are essential for certain metabolic processes involved in growth.

B. *Effects of Other Types of Toxicants*

The vast majority of the fungicides that are used in the practical control of plant diseases appear to be capable of much more generalized effects on metabolism than are the analogues of metabolites. Results of research in recent years indicate that many of them are potentially capable of reacting with functional chemical groups of enzymes and coenzymes, with metal activators, or other components of metabolic systems, many of which are common to various aspects of metabolism. This being the case, it does not seem practical to separate metabolism into various categories such as energy release, protein synthesis, etc., and discuss how fungicides affect each of them. It does seem to the point, however, to discuss the manner in which several representative types of fungicides are reported to affect cell metabolism and enzymes. It is also appropriate to mention here some of the possible explanations of how these fungicides may be used to control pathogens without injuring the higher plants to which they are applied.

The activity of quinones against diverse types of enzymes suggests that they are capable of blocking cellular metabolism at a number of stages. Quinones are effective inhibitors of pancreatic amylase, an amino-dependent enzyme, and of malt amylase, a sulfhydryl-dependent enzyme and they are also moderately inhibitory to catalase (Owens, 1953). A number of the quinones are potent inhibitors of carboxylase (Kuhn and Beinert, 1947; Foote *et al.*, 1949; Hochstein and Cox, 1952). In fact, their effectiveness as inhibitors of carboxylase was sufficiently correlated with their toxicity to spores of *Monilinia fructicola* that Foote *et al.* (1949) were led to suggest that *in vitro* carboxylase inhibition might be used as a preliminary screen for selecting quinones for use as fungicides. A number of other enzymes, including urease, pancreatic lipase, proteinase, and phosphatase, are also inhibited by certain quinones (cf. McNew and Burchfield, 1951).

Dichlone, which is 2,3-dichloro-1,4-napthoquinone, reacts with coenzyme A *in vitro* and it was suggested that its extensive effects in disrupting cellular metabolism in *N. sitophila* may result from its blocking of many reactions in cells that require coenzyme A (Owens, 1957). The concentrations of chloranil and of dichlone that are required to inhibit 50% of the oxygen consumption by conidia of some species of fungi are essentially equivalent to the amounts required to prevent 50% of their germination, but other fungi require greater quantities to inhibit oxygen uptake by 50% than are required to give an equivalent inhibition of germination (McCallan *et al.*, 1954). This suggests that retardation of growth in the two cases may result from effects on different phases of metabolism. Some degree of selectivity for a particular phase of metabolism within sensitive cells might be expected as a result of variations in enzyme sensitivity or of the distribution of the toxicant within the cell, but it seems most likely that a number of metabolic processes would be affected with almost equal facility by growth-inhibiting concentrations of quinones. Since the quinones are capable of combining with both –SH and amino groups, of oxidizing –SH groups, and of changing redox potentials (McNew and Burchfield, 1951), their selectivity toward various organisms, as Horsfall (1956) has concluded, probably rests mainly on two factors—permeation and detoxification. Evidence presented by Rich and Horsfall (1954) indicates that polyphenol oxidases in fungi may be significant in detoxifying phenols and quinones.

At concentrations sufficient to prevent germination and growth, captan, *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide, virtually eliminates uptake of oxygen by conidia of *M. fructicola* and it is as effective in reducing consumption of oxygen by spores of *Alternaria solani* as it is in suppressing their germination (McCallan *et al.*, 1954). Oxygen uptake by dense suspensions of conidia of *F. roseum* is severely reduced by captan at a concentration of 10^{-4} moles per liter. Hochstein and Cox (1956) showed that pyruvate accumulates in cells of this fungus when they are exposed to captan and that yeast carboxylase activity *in vitro* is inhibited. Thus, it appears that effects on early stages of metabolism (assimilation of substrate and carbohydrate oxidation) may be sufficient to account for toxicity of captan to some fungal cells. Its effects are not limited to fungi. Captan is toxic to cells of higher plants if it gains access to them in sufficient concentration. It is quite toxic to bean and tomato plants even at low concentration (1–5 p.p.m.) when it is applied to the roots in liquid culture (Silber, 1957; Lukens and Sisler, 1958). Apparently it is capable of affecting metabolic systems in some higher plants similar to those that it affects in fungi because Dugger *et al.* (1959) have shown that it causes accumulation of pyruvate in pea root tips and that it strongly inhibits pyruvic oxidase and α-ketoglutaric oxidase activity in preparations of mitochondria from lupine plants. They also reported that captan inhibits hexokinase from yeast and the oxidation of ribose-5-phosphate by extracts from peas. Lukens and Sisler (1958) have presented evidence to support the contention that captan owes its ultimate toxicity to the reactive $-SCCl_3$ group that is released or to thiosposgene that is formed following cleavage of the captan molecule by sulfhydryl groups in commonly occurring cellular components. One would not expect a high degree of specificity from these reactive derivatives. It would appear, therefore, that such specificity as captan possesses must be attributed to differences in its ability to penetrate and accumulate in cells and possibly also to differences in the facility with which cells of different species are able to release the $-SCCl_3$ group from the imide portion of the molecule.

An understanding of the way in which such dialkyldithiocarbamate fungicides as thiram (tetramethylthiuram disulfide), ziram (zinc dimethyldithiocarbamate), and their relatives affect cellular metabolism has been complicated by the tendency of these fungicides to be less toxic when used at certain intermediate concentrations than when used at either lower or higher concentrations. Dimond *et al.* (1941) first reported this kind of behavior for thiram and it has since been observed by a number of investigators with thiram and related compounds. The range of intermediate concentrations at which growth of fungi occurs has been designated by Sijpesteijn and van der Kerk (1956) as the zone of inversion growth. Occurrence of this phenomenon appears to be regulated by the concentration of certain heavy metals in the medium or in association with the cells (Goksøyr, 1955; Smale, 1957; Sijpesteijn *et al.*, 1957). With *Saccharomyces pastorianus* as the test organism, the zone of inversion growth characteristic of sodium dimethyldithiocarbamate was almost completely abolished by addition of certain trace metals in sufficient quantity to the medium in which the yeast was grown. Zinc appears to be the most critical of the metals in this case (Smale, 1957). Areas of metabolism affected by concentrations of fungicide on the lower side of the zone of inversion growth are probably different from those affected by toxic doses on the uper side of the zone of inversion growth.

α-Ketoglutaric acid accumulates in the mycelium of *F. roseum* when it is treated with ziram (Sisler and Marshall, 1957) and pyruvic acid accumulates in cultures of *A. niger* and of *Penicillium italicum* when certain concentrations of sodium dimethyldithiocarbamate are present (Sijpesteijn and van der Kerk, 1956). α-Keto acids antagonize the toxicity of this fungicide toward *A. niger*, but they do not offset its toxicity to certain other fungi. It was thought originally that the pyruvic acid that accumulated in the presence of the fungicide might play an important role in initiating the phenomenon of inversion growth, but it now appears that this is not the case (Sijpesteijn *et al.*, 1957).

Accumulation of pyruvate and α-ketoglutarate, which are intermediates of carbohydrate metabolism, suggests that dithiocarbamates affect respiration, and such an effect has been observed. Thiram suppresses oxygen uptake, carbon dioxide production, and germination of conidia of *F. roseum* (Sisler and Cox, 1954), and of *Myrothecium verrucaria* (Walker, 1955). Thiram's close relative, antabuse (tetraethylthiuram disulfide), prevents growth of *Staphylococcus aureus* at quite low concentrations and inhibition of growth is directly correlated with suppression of respiration (Balestrieri, 1952). At concentrations that prevent growth of *F. roseum*, ziram suppresses respiration by about 50%, but higher concentrations have no further effect on respiration (Sisler and Marshall, 1957). McCallan *et al.* (1954) showed that respiration of conidia of *Monilinia fructicola* is more sensitive to ferbam than is their germination, but with spores of some other species of fungi the opposite is true. At a concentration that prevents growth of *Saccharomyces cerevisiae*, sodium dimethyl dithiocarbamate was demonstrated by Goksøyr (1955) to depress oxidation of glucose by about 20%, but oxidation of acetate is somewhat more inhibited than is that of glucose.

These examples serve to demonstrate that at concentrations which are toxic to fungi, dithiocarbamates often affect respiration, but that the extent of the effect and the relationship between suppression of respiration and suppression of germination of spores and growth of fungi varies with the particular fungicide and with the species of fungus that serves as the test organism. There is not now sufficient evidence in the case of any of the dithiocarbamates to indicate conclusively that inhibition of growth of fungi is the direct result of suppression of respiration. In those cases where growth-inhibiting concentrations have little or no effect on respiration it would seem to be a safe assumption that the primary basis of toxicity is to be sought elsewhere.

When it was discovered that the toxic effect of some of the dialkyldithiocarbamates is overcome in part by addition of histidine or of certain other imidazole derivatives, it was suggested that they may owe their toxicity to interference with synthesis of histidine or with some vital process in which it is required (Sijpesteijn and van der Kerk, 1952). However, since cysteine is also somewhat antagonistic and, like histidine, is a chelator, it now appears that the protection that they afford is most probably related to their ability to compete for heavy metals involved in toxicity of the dithiocarbamates.

There is a considerable literature on the effects of dialkyldithiocarbamates on enzymes from various sources. Among the enzymes reported to be affected are: copper-containing enzymes such as catechol oxidase, laccase, and ascorbic acid dehydrogenase (cf. Goksøyr, 1955); the sulfhydryl enzymes—aldehyde dehydrogenase, triosephosphate dehydrogenase (Graham, 1951; Nygaard and Sumner, 1952; Sisler and Cox, 1955), and succinoxidase (Keilin and Hartree, 1940). In addition, Owens (1953) demonstrated that pancreatic amylase, malt amylase, polyphenoloxidase, and catalase, which respectively represent amino-, sulfhydryl-, copper-, and iron-dependent enzymes are all inhibited by dithiocarbamic acid derivatives. Thus, it is apparent that these fungicides possess the potential of interfering with a number of metabolic processes. However, the fact that they inhibit a wide variety of enzymes when isolated in the laboratory does not necessarily imply that they owe their toxic action to effects on these enzymes in the intact cell. This is illustrated by Goksøyr's (1955) observation that certain dithiocarbamyl fungicides are strongly inhibitory to yeast succinoxidase *in vitro*, but have little or no effect on oxidation of succinate by yeast cells.

Goksøyr's (1955) extensive studies of the effects of dithiocarbamyl compounds on S. *cerevisiae* indicated that in intact cells the major areas of metabolism, such as respiration, protein synthesis, and synthesis of nucleic acids, are not primarily affected by the dithiocarbamates. He concluded that these fungicides most probably act at the surface of cells, affecting in varying degrees a rather large and diverse group of enzymes at that location. It remains to be seen whether this is the case for fungi in general, in view of the reports of the accumulation of keto acids in cells treated with these toxicants. Goksøyr finally concluded that anatomical differences between cells of different organisms must play a more important role than do physiological differences in determining selectivity of the dialkyldithiocarbamyl fungicides.

The bisdithiocarbamates, the best known fungicidal representatives of which are nabam, zineb, and maneb, apparently operate by mechanisms that differ from those of the dialkyldithiocarbamates. Presently available evidence indicates that they are converted to isothiocyanates that inactivate essential —SH systems of cells (Sijpesteijn and van der Kerk, 1954).

Turning our attention to a relatively new group of fungicides, the -*s*-triazine derivatives, we find that they possess the potential of arresting a variety of vital biochemical reactions in cells (Burchfield and Storrs, 1956, 1957). It was shown that 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine, for instance, will combine with amino acids, glutathione, para-aminobenzoic acid, proteins, pyridoxine, pyridoxamine, and niacin. In view of these capabilities, it was concluded that any selectivity that these compounds may demonstrate with regard to sites of action in cells is probably dependent upon relative reaction rates rather than upon absolute specificity for a single receptor site. Since they are reactive with compounds common to all organisms, the decisive factors regulating species specificity are probably the degrees to which they are accumulated in cells and the internal pH of the cells since there is approximately a tenfold decrease in the reactivity of these compounds for each unit drop in pH.

Cycloheximide—possibly the best known of the antifungal antibiotics—is a fungitoxicant that is apparently considerably more specific in its mode of action than are most of the synthetic organic fungicides. However, its selectivity toward species is no better and perhaps much poorer than that of many of the organic fungicides. Sensitivity to this antibiotic is widely distributed among organisms, as is evidenced by its toxicity to higher plants and animals, fungi, and protozoa, and yet it possesses a most remarkable specificity for certain closely related species. For example, growth of some species of yeasts is absolutely prevented by 0.17 p.p.m. of cycloheximide, whereas under the same conditions other species grow even in the presence of 1000 p.p.m. (Whiffen, 1948). It inhibits germination of conidia of some fungi without any effect on oxygen uptake (McCallan *et al.*, 1954). Respiration in other species is markedly affected. For example, concentrations that prevent germination of spores of *Myrothecium verrucaria* reduce their oxygen consumption by about 85% (Walker and Smith, 1952). With *F. roseum*, oxygen uptake is depressed about 50% by concentrations that prevent growth, but even much higher concentrations do not significantly increase the effect on respiration (Sisler and Marshall, 1957). This antibiotic brings about a marked reduction in endogenous respiration and interferes with oxidation of sugars, amino acids, and organic acids by the protozoan *Tetrahymena pyriformis* (Mefferd and Loefer, 1954).

The mode of action of cycloheximide is not presently understood and, while it is known to induce mitotic disturbances in some organisms (as is pointed out later in this chapter), it seems unlikely in view of its effects on metabolism that its toxicity can be accounted for entirely in this way.

Normally, one would expect enzymes or proteins at the outer surface of the cell to be affected by ions of the heavy metals, but they most probably react with labile chemical groups in cellular components whereever they come in contact with them. The lack of specificity of these fungicides is indicated by the fact that they are inhibitory to a wide variety of enzymes. The enzyme they attack in the living cell may depend on its location in the cell. There are essentially no grounds for assuming that the enzymes of pathogen or host cells or of cells of different pathogens would be markedly different in their tolerances to heavy metals such as copper, mercury, and silver. Any tolerance that cells may have for these toxicants would probably rest on the nature of the sensitive groups that are exposed at the surface of the cytoplasm, on their ability to exclude them from the cytoplasm, or on their ability to bind them into nontoxic complexes.

The mechanism of cytotoxic action, defined in terms of interference with a specific biochemical reaction, has not been established conclusively for any of the common fungicides. The problem of establishing a specific basis for cytotoxicity is complicated by several factors. For reasons that have been indicated, the particular reactions or processes that are affected by a given toxicant may not be the same in all susceptible species even though the chemical basis for toxicity is the same. The chemical reactivity of most of the fungicides suggests that they may often affect several processes simultaneously and to about the same degree. Moreover, they may bring about secondary effects that tend to obscure less obvious primary effects.

C. *Accumulation of Toxic Metabolites and Enzyme Stimulation or Induction*

It is conceivable that a fungitoxicant might interfere with metabolism in such a manner that toxicity results directly from the effects of an accumulated metabolite. While most metabolites would not be harmful except in relatively large quantities, hydrogen peroxide is an example of one that would be detrimental even in small quantities. It has been demonstrated that sublethal doses of cyanide increase the mutation rate in *Neurospora* and this effect is attributed to hydrogen peroxide accumulated as a result of inhibition of catalase by the cyanide (Wagner *et al.*, 1950). Similar results have been obtained with cells of *E. coli* treated with azide (Wyss *et al.*, 1948). Lethal effects from peroxide might well result if a fungicide exerted a sufficiently inhibitory effect on catalase.

As opposed to inhibition of enzymes, stimulation of an enzyme into abnormal activity would constitute a mechanism of toxicity. It has been postulated that the effect of dinitrophenol on oxidative phosphorylation may be the result of its activation or stimulation of ATP-ase in the mitochondria (Hunter, 1951).

Perhaps one of the most effective and subtle means of killing a fungus cell would be to induce its enzymatic self-destruction. Prestidge and Pardee (1957) presented evidence that penicillin induces formation of a lytic enzyme in cells of *E. coli* that attacks the cell membrane. Compounds like chloramphenicol and 7-azatryptophan, that inhibit synthesis of enzyme protein, protect cells from penicillin.

It should be recognized that the ultimate cause of death of fungus cells, in which normal metabolism is disrupted by a fungicide, may in many cases be a form of autolysis in which cellular components are irreversibly altered by enzymes.

VII. Biological Modification of Fungicides

Susceptibility of an organism to a fungicide may in some instances depend on whether it can activate or detoxify the fungicide. If a compound is modified by an organism its toxicity may be increased, decreased, or remain unaltered. Generally, mechanisms for altering foreign chemicals involve metabolic systems in existence in the cells prior to their exposure to a toxicant (Sexton, 1953), but there are exceptions to this generalization. Penicillinase accounts for the resistance of certain bacteria to penicillin. Development of penicillinase is an adaptive process in some species (Pollock and Perret, 1951). Horsfall (1956) cited instances in which appreciable periods of association between fungi and fungicides are required before they are able to overcome the toxic effects, which suggests that detoxification awaits development of detoxifying mechanisms in the cells. Activation or detoxification may involve direct enzymatic action on the toxicant or, in other cases, they may involve combination of the toxicant with metabolites or other cellular components. Conceivably, a toxicant may remain unaltered, but nevertheless be rendered ineffective in a particular biological system by a competitive metabolite the accumulation of which is incited by the toxicant.

A. *Activation*

Although biological activation has received less attention than detoxification, it may be a significant factor in controlling toxicity and specificity of certain fungicides, insecticides, and growth regulators. Toxicity of organic phosphorus insecticides is generally attributed to interference with cholinesterase activity (Fukuto and Metcalf, 1956). Preliminary *in vivo* oxidation is required to convert some members of this group into active cholinesterase inhibitors (Casida, 1956). Beta oxidation of the side chains of certain phenoxyalkylcarboxylic acids by plant tissues is reported to be necessary for their conversion into active herbicides (Wain, 1955). Different species of higher plants vary in their ability to bring about this oxidation, which probably accounts for species specificity of these herbicides.

Dichlone (2,4-dichloro-1,4-naphthoquinone), the corresponding naphthohydroquinone, and its diacetyl ester are equally toxic when applied to certain fungi. Byrde and Woodcock (1953) presented good evidence that the diacetyl ester is biologically activated by conversion, through hydrolysis, to naphthohydroquinone, and Horsfall (1956) postulated that a phenol oxidase finally converts it to the toxic quinone form. The importance of biological modification of dialkyldithiocarbamate fungicides is not entirely clear, but it is apparent, as is indicated by the following examples, that these fungicides are subject to alteration by fungi. Cells of *F. roseum* decompose thiram with release of CS_2 (Sisler and Cox, 1954). This gas apparently arises from acid decomposition of dimethyldithiocarbamate ions formed as a result of biological reduction of the thiram. Goksøyr (1955) demonstrated by spectrophotometric means that cells of baker's yeast can reduce thiram, and Johnston (1953) showed that thiuram disulfides can be reduced by glutathione. On the other hand, Keilin and Hartree (1940) found that the cytochrome oxidase system in homogenates of animal cells oxidizes sodium diethyldithiocarbamate to antabuse which strongly inhibits the succinoxidase system, and Neufeld *et al.* (1957) prepared an extract from *Piricularia oryzae* that performs the same kind of oxidation. Biological oxidation and reduction of the dialkyldithiocarbamates may possibly be of importance in regulating their toxicity, or such reactions may simply be examples of metabolism of foreign chemicals in which toxicity is not significantly altered.

Sproston (1957) described an interesting case of activation associated with the natural resistance of *Impatiens balsamina* to fungal infection. The fungitoxicants in this case apear to be polyphenolic compounds that occur in the plant cells probably as glycosides or other bound forms. When spores of any of several fungal species germinate on the leaves and the hyphae penetrate the epidermal cells, the invading fungi apparently free or otherwise activate the polyphenols with the result that both the invaded plant cells and the fungus are killed.

B. *Detoxification*

Inherent detoxification mechanisms may account for the insensitivity of some species of fungi to toxicants that destroy other species. For example, strains of *Endomyces vernalis* that are resistant to pyrithiamine decompose it, whereas sensitive strains do not (Woolley, 1944). Desthiobiotin strongly inhibits growth of *Lactobacillus casei* but actually has biotin activity for a number of yeasts and filamentous fungi (Dittmer *et al.*, 1944; Lilly and Leonian, 1944). The latter organisms are apparently able to insert sulfur into the molecule thus converting it to biotin.

Quinones and phenols are fungitoxic compounds which may be subject to detoxification by fungal cells. In a study of toxicity of 44 phenols and quinones, Rich and Horsfall (1954) showed that those compounds which were oxidized and polymerized to yield colored products, when exposed to an extract from the mycelium of *Stemphylium sarcinaeforme*, are usually nontoxic to spores of the fungus. Of the compounds that yield a colored product 88% are nontoxic, while of those that do not yield a colored product 84% are toxic. A similar but less striking relationship exists between toxicity and ability of mycelial extracts of *Monilinia fructicola* to oxidize and polymerize quinones and phenols.

Aspergillus niger detoxifies 2-naphthyloxyacetic acid and β-(2-naphthyloxy)propionic acid by hydroxylating them at the 6- position (Byrde *et al.*, 1956) and reduces the toxicity of phenoxyacetic and β-phenoxypropionic by hydroxylating them at either the *para* or *ortho* position on the benzene ring (Byrde and Woodcock, 1957).

Some fungi tolerant of arsenic metabolize it to volatile trimethyl arsine (Challenger, 1945) and this may represent a detoxification mechanism, but there are other species with high tolerance that do not metabolize arsenic in this manner.

Certain metabolites already present in fungal cells at the time of treatment undoubtedly contribute to protection of vital cellular components by reacting with fungitoxic compounds. Many amino acids will antidote the toxicity of copper sulfate to *S. sarcinaeforme* and *M. fructicola* (Adam and Powell, 1957). A number of fungicides interfere with sulfhydryl groups of proteins and coenzymes, and naturally occurring sulfhydryl compounds such as cysteine and glutathione protect the proteins and coenzymes by combining with the toxicant and rendering it ineffective. Sulfhydryl compounds are known to react with heavy metals, with nabam (Sijpesteijn and van der Kerk, 1954), with captan (Lukens and Sisler, 1958), with -*s*-triazines (Burchfield and Storrs, 1957), and with quinones (cf. McNew and Burchfield, 1951) to form nontoxic derivatives.

Since detoxification often makes use of essential metabolites and of normal metabolic machinery, it should be expected that depletion of metabolites or overloading of the systems might ultimately result in death of the cells. The case of sulfur is a good example. Its toxicity to fungal cells is lowered when they metabolize it to hydrogen sulfide, but the process of detoxification, i.e., reduction, is thought to be responsible for death of the cells (Miller *et al.*, 1953a). In animals, cysteine is involved in the detoxification of bromobenzene. Large doses of bromobenzene cause cessation of growth because there is insufficient cysteine for both detoxification and growth (Williams, 1947). Supplies of cellular components capable of inactivating toxicants are limited and can confer only a limited degree of resistance to a fungicide. However, the relatively large quantities that are required of many common fungicides on a spore weight basis to inhibit germination have been attributed to more or less indiscriminate interaction between toxicant and various cellular components (Owens and Miller, 1957). For instance, at subinhibitory concentrations dichlone reacts with substances from conidia of *N. sitophila* to form at least five different products (Owens and Miller, 1957). If any of the reactants are vital metabolites, they occur in the spores in sufficient quantities to detoxify appreciable quantities of dichlone with enough remaining to support germination. It is apparent that one of the characteristics of a fungicide of high efficiency (on a microgram-per-gram basis) must be that of discriminate reactivity with cellular components.

Detoxification by reaction with a nonvital cellular component occurs in certain wood-destroying fungi that produce oxalic acid. In these organisms resistance to copper fungicides results from formation of the relatively nontoxic copper oxalate (Rabanus, 1939).

VIII. Acquired Resistance to Fungicides

Sustained, acquired resistance to fungicides in populations of plant pathogens has not developed into a problem comparable to that of acquired resistance to antibiotics in bacterial populations or of acquired resistance in insects to certain organic insecticides. Reports of fungicide-resistant strains of plant pathogens in the field are limited. Taylor (1953) collected spores of *Physalospora obtusa* from a number of orchards and found greater tolerance to Bordeaux mixture in those from orchards that had been continuously sprayed with that fungicide than in those from orchards not so sprayed. There is abundant evidence from the laboratory, however, that fungi either have the ability to develop tolerance to diverse kinds of fungicides or else that natural populations contain a few potentially resistant individuals that gain the ascendancy in the population when the organisms are cultivated in the presence of the toxicant. Whiffen (1948) found that *Saccharomyces pastorianus* acquired a sixteenfold increase in tolerance to cycloheximide after three transfers in medium containing the antibiotic, and Roper and Käfer (1957) isolated, from a medium containing acriflavine, three strains of *A. nidulans* that were resistant to this toxicant. In each of the three strains resistance was due to single gene mutation.

There are reports of strains of fungi that are more tolerant than the normal population to arsenic (Stakman *et al.*, 1946; Wilson, 1947), to thiram and organic mercury (Gattani, 1951), to tetrachloronitrobenzene (McKee, 1951), and to copper sulfate (Mader and Schneider, 1948; King and Keplinger, 1951). In some of these cases the fungi retained their tolerance when cultivated in the absence of the fungicide, which indicates a permanent change, whereas others lost their tolerance. Leben *et al.* (1955) exposed conidia of *Venturia inaequalis* to ultraviolet light, but did not find among the survivors mutants that were resistant to thiram or to sulfur.

A factor that may account, in part, for failure of resistant strains of pathogens to develop in the field may be the nature of the environment in which spores of most plant pathogens are exposed to fungicides. Leaf surfaces are relatively poor in substrates and there is good evidence that resistant strains are less likely to develop when certain nutrients are in limited supply or are unavailable. This is suggested by the more frequent reports of resistance arising in culture medium in the laboratory than in the field. Sevag and Rosanoff (1952) noted that when either aspartic acid or phenylalanine are absent from a medium that contained streptomycin, resistant strains of *Micrococcus* fail to emerge from the population, although strains of the bacterium, which have developed resistance in the presence of these amino acids, retain their resistance to streptomycin when grown in medium lacking aspartic acid and phenylalanine. Schnitzer and Grunberg (1957) discuss other instances in which emergence of forms of bacteria that are resistant to toxicants is dependent on the composition of the medium. Mechanisms of resistance that involve utilization of detoxifying metabolites or changes in pH may be practically inoperative in an environment of limited exogenous substrate.

Another, and likely more important, factor in preventing emergence of fungicide-resistant forms in the field is the less specific mode of action of most fungicides as compared to those of the antibiotics and of some of the insecticides. When a toxicant can affect a number of vital processes, any one of which may inhibit growth, there is less likelihood that resistant strains of an organism will develop than when toxicity is based on more specific effects. If development of resistance is dependent on gene mutation, one would expect that strains of pathogens resistant to mutagenic fungicides would be more prevalent than those resistant to fungicides which are not mutagens, but apparently this possibility has never been investigated.

As fungicides that are more complex chemically and more specific in their modes of action come into general use, the problem of resistance of pathogens to fungicides in the field is likely to arise.

IX. MUTATION, MITOTIC DISTURBANCES, AND MORPHOLOGICAL ABERRATION

A. *Mutagenesis*

A number of fungitoxic compounds produce mutations or affect the process of mitosis. In considering the physiology of fungitoxicity one should be concerned with mutagenic effects both because they may be involved in the mechanism of action of the fungicides and because of any possible hazard that they may constitute for people who handle or who are otherwise exposed to such toxicants. Mutagenic chemicals are usually recognized by the heritable abnormalities that they produce in the progeny of those cells that survive treatment. When cells do not survive treatment with a fungicide, the cause of death might well be lethal mutation without being recognized as such.

In addition to gene mutations, there may be cytological mutations involving structural changes in chromosomes such as breaks, deletions, and inversions. Inasmuch as chromosomes of most fungi are too small for easy cytological study, much of the evidence for fungicidal effects on chromosomes and mitotic behavior is indirect and is based on observable effects in root tips of *Vicia faba* and *Allium cepa*. Toxic chemicals sometimes cause pycnosis or stickiness of chromosomes. In the most severe instances, the chromosomes appear to melt together and recovery of cells so affected is impossible (Levan, 1951). Some chromosomal aberrations may not arise from direct action of a toxicant, but rather may represent post lethal autolytic damage, possibly involving the action of deoxyribonuclease or of proteolytic enzymes.

Quinones, phenols, and 8-hydroxyquinoline are among the fungitoxic compounds reported by Levan (1951) to be mutagenic in root tip tests with higher plants. The fungicide, tetrachloronitrobenzene, induces mutations in *F. coeruleum* (McKee, 1951). Fries and Kihlman (1948) produced mutations in *Ophiostoma multiannulatum* with certain methyl xanthines. The mutations produced by chemicals of this type may involve interference with nucleic acid metabolism (Loveless and Revell, 1949). It seems possible that these purine analogues may replace normal components and result in the synthesis of defective nucleic acids. This would represent a case of lethal synthesis for those cells that do not survive. Mutagenesis may result from accumulation of toxic metabolites. Compounds that poison catalase, which normally destroys hydrogen peroxide in cells, bring about an increase in the rate of mutation in *Neurospora* (Wagner *et al.*, 1950) and in bacteria (Wyss *et al.*, 1948).

Some of the same fungitoxicants that are known to induce lethal mutations, drastic structural changes in chromosomes, or abnormal chromosomal behavior are capable also of other effects in cells. In such cases, it may be difficult to determine which effect is primarily responsible for their general toxicity to fungal populations. An excellent example is presented by the nitrogen mustards which are well-known mutagens, but which also inactivate the enzymes, hexokinase, adenosine triphosphatase, choline, oxidase, and acetylase (Sizer, 1957). Herriott (1948) found, however, that sulfur mustards inactivate viruses, especially those that contain deoxyribonucleic acid, more readily than they inactivate enzymes. He suggested that this was the result of reaction with nucleic acid. If this is true also of the nitrogen mustards, it constitutes strong circumstantial evidence that their primary toxic effects on cells may involve the chromosomes. Formaldehyde causes mutations in *Neurospora* as well as in higher plants, bacteria, and *Drosophila* (Jensen *et al.*, 1951). Its wide use in inactivating viruses is ample evidence of its activity against nucleoproteins. Cartwright *et al.* (1956) suggested a number of possible sites of lethal action of formaldehyde with the tobacco mosaic virus. Included are the amino groups of the purines and pyrimidines and the hydroxy groups of ribose in the nucleic acid. These appear to be the most likely sites of action, because it is generally conceded that the nucleic acid portion of the virus contains the biologically active centers. Viruses and chromosomes are similar in their chemical composition. Thus with formaldehyde, as with the mustards, there is reason to suspect that the primary lethal effect may be on the chromosomes, but this need not necessarily be the case because a number of vital components other than nucleic acids of the chromosomes are reactive with formaldehyde and in many cases may be more accessible to the toxicant.

B. *Interference with the Mitotic Spindle*

In the process of cell division the orderly movement of the two sets of chromosomes to opposite poles of the dividing cell involves the formation and functioning of the mitotic spindle. Appearance of the spindle mechanism in some cells is accompanied by abrupt changes in the viscosity of the protoplasm—sometimes referred to as mitotic gelation—and the spindle fibers appear to crystallize from the protoplasmic fluid (Heilbrunn, 1952). They are similar in appearance to tactoids or liquid crystals and their structure may involve hydrophobic linkages between proteins or between proteins and lipids. Malformation or malfunction of the spindle results in polyploidy, unequal distribution of nuclear material, or other cytological aberrations. Such phenomena are sometimes initiated by fungicides. In general, fungicides that affect development or function of the mitotic spindle differ from those that affect chromosomes directly (Horsfall, 1956).

One major group of compounds that affect spindle formation is the lipid-soluble compounds whose activity is correlated with their physical properties rather than with their chemical reactivity (Levan and Östergren, 1943). Included in this group are naphthalene and benzene and their derivatives and most probably many other compounds of this type. Fat-soluble anesthetics and colchicine, at concentrations that prevent cell division, typically prevent mitotic gelation in sea urchin eggs (Heilbrunn, 1952). Compounds of this kind probably exert their effects through interference with hydrophobic linkages in the spindle structure.

Organic mercury compounds constitute a second group with rather high activity in disrupting spindle function, but they probably act by a different mechanism from the previous group of compounds. Levan (1951) reported that butyl mercury iodide is about 1,000 times more effective than is colchicine in inducing c-mitosis in onion root tips. Organic mercury fungicides, used as treatments on rye seed, have been known to induce tetraploidy (Levan, 1951).

Cycloheximide consistently produces aberrant mitotic behavior in onion root tips (Wilson, 1950) and affects meiosis in *Gymnosporangium* in a manner suggesting malfunctioning of the spindle (Berliner and Olive, 1953). A list of compounds that are reported to produce mitotic aberrations has been compiled by Horsfall (1956).

C. *Effects on Morphology*

Morphological abnormalities, such as giant cells, hyphal monstrosities, and a filamentous habit in organisms that normally grow by fragmentation into individual cells, are among the effects produced in plant pathogens, other fungi, and bacteria by certain toxic agents. Responses of this kind imply interference of such a nature that the cell, although unable to divide normally, retains its capacity for growth—at least for a while. Certain specific inhibitors of cell division characteristically induce bacteria to grow in filamentous form, and yeast to produce enlarged paired cells (Loveless *et al.*, 1954).

Although general interference with mitosis may in some cases account for abnormalities of the kind under discussion here, there are other instances in which there is a more specific effect, posibly involving dissociation of the processes of nuclear division from cleavage of the cytoplasm or of the cell wall. Nickerson (1948) advanced the hypothesis that cell division normally involves a chain of enzymatic reactions that can be uncoupled from growth processes. It is thought that sulfhydryl-containing substances play a role in fragmentation, because such compounds as cysteine and glutathione promote cell division and antagonize the effects of such substances as cobalt and penicillin that tend to prevent cell division.

Horsfall (1956) has compiled and discussed much of the literature on toxicants that incite morphological abnormalities in fungi.

Allison and Christensen (1957) described the injurious effects of the antibiotic, filipin, on *Helminthosporium sativum* as including mutations, tumorlike growths, and rupturing of the cells with extrusion of the protoplasts. Among the chemicals that inhibit cell division in *E. coli* without inhibiting growth are: acriflavine, diazouracil, diethyl sulfate, ethylenimine, 5-nitro-7-hydroxybenzothiazole, and β-propiolactone. Nitrogen mustard and triethylenemelamine specifically inhibit cell division in baker's yeast (Loveless *et al.*, 1954). Morphogenic effects are also produced by appropriate doses of irradiation. Ultraviolet treatment of *E. coli* results in its developing in the form of "long filaments" or "snakes." The effect appears to be quite specific since syntheses of deoxyribonucleic acid, ribonucleic acid, and proteins continue at the same rates in irradiated as in unirradiated cells (Deering and Setlow, 1957). At proper levels, α-, γ-, and X-irradiation also inhibit cell division without affecting growth of *E. coli* and of *S. cerevisiae* (Loveless *et al.*, 1954). It is interesting that many of the agents that specifically inhibit cell division are also mutagenic, but there is no definite evidence that the failure of cells to divide after treatment with these agents is a result of damage to chromosomes. Death ultimately results if cells fail to regain their ability to divide normally.

X. Spores versus Mycelium

A. *Effects on Sporulation*

Sporulation in filamentous fungi is a fragmentation process that apparently does not differ in any fundamental way from the behavior of bacteria and yeasts that normally reproduce by fragmentation into individual cells. The process may be considered as consisting of two steps—mitosis in which nuclear material is distributed properly between the mother and daughter cells and, second, cell division involving cleavage of the cytoplasm and organization of new cell wall material in such a way that the spore separates from the structure on which it is produced.

A number of fungus diseases of plants could be controlled effectively if sporulation could be prevented so as to reduce the inoculum potential. Horsfall and Rich (1955) became interested in compounds that might selectively inhibit sporulation as a possible means of plant disease control. They compared a number of compounds, including mitotic poisons, chelating agents, and others as to their relative effectiveness in reducing mycelial growth and in inhibiting production of conidia by *Monilinia fructicola*. Some of the mitotic poisons showed greater inhibitory activity toward sporulation than toward mycelial growth, as did also a number of the chelating agents. Several compounds, including ketones, acids, and amides that are α-β unsaturated, actually enhanced sporulation, while among the compounds that were most active in selectively suppressing sporulation in these experiments were sodium thiocyanate, benzidine hydrochloride, diphenylthiocarbazone, and bis(4-dimethylaminophenyl) methane. Metals may promote sporulation (fragmentation) in some instances (Foster, 1949; Bortels, 1927), but in others have the opposite effect. Cobalt, for example, induces certain yeasts to grow in filamentous form (Nickerson, 1948). Zinc is involved in the fragmentation process in cultures of *Ustilago sphaerogena* (Grimm and Allen, 1954; Spoerl *et al.*, 1957), but the exact nature of involvement is not understood. Compounds that react with metals might logically be expected to affect sporulation and Rich and Horsfall (1948) found that the metal chelator, dimethylglyoxime, prevents sporulation in *A. niger* at concentrations that do not appreciably affect growth of mycelium. Inhibition of sporulation in *A. niger* by thiourea (Fleury, 1948) and in *Peronospora destructor* by limesulfur (Yarwood, 1937) is considered to result from interference with essential metals (Horsfall, 1956).

It is well known that sporulation of many plant pathogenic fungi in culture is sensitive to minor nutritional and environmental changes that do not appreciably affect mycelial development. If sporulation in nature is likewise a more sensitive process than growth, it would appear to be appropriate to continue and to intensify the search for specific chemical inhibitors of sporulation, because reduction of inoculum potential might constitute an easier and more efficient means of controlling some plant diseases than attempting to suppress spore germination and infection by use of protectant fungicides.

B. *Comparative Sensitivity of Spores and Mycelium*

Some toxicants are more effective in inhibiting spore germination than they are in inhibiting mycelial growth, whereas other toxicants exhibit the opposite effect. Diphenyl, for example, does not inhibit germination of spores of several species of fungi, but it is inhibitory to hyphal growth of these organisms (Ramsey *et al.*, 1944; Horsfall, 1956). Spores of the fungus *Trichophyton purpureum* germinate in solutions containing sufficient sulfanilamide to completely inhibit hyphal growth (N. S. Dimond, 1941). Horsfall and Rich (1953) reported a number of compounds that inhibit mycelial growth of *M. fructicola,* but fail to prevent spore germination.

Since germination does not necessarily involve nuclear division or require the transport of substrates into the cell, as is the case in mycelial growth, it has been proposed that interference with these processes could logically account for the greater sensitivity of hyphae (Horsfall, 1956). Spores ordinarily carry greater reserves of certain vital metabolites than mycelium and interference with the synthesis of these would not become apparent during germination, but would be immediately apparent in hyphal growth.

Higher sensitivity of spores than of mycelium, may be explained in some cases by the relatively slow rate at which mycelium accumulates toxicants from the medium (McCallan and Miller, 1957). However, they showed that on the basis of the ultimate quantities of toxicant taken up, spores and mycelium are about equally sensitive. When interpreting observed differences in sensitivity between spores and mycelium, one must consider whether the amount of fungal material was equivalent in the two cases.

A criticism that might be leveled at the spore germination test for evaluating fungicides is that it fails to detect toxicants which inhibit mycelial growth without affecting germination of spores.

XI. CONCLUSION

Considerable progress has been made in identifying the cellular activities that are involved when plant pathogenic organisms are controlled by fungicides and in recognizing the factors governing sensitivity and resistance to toxic compounds. Our knowledge of cellular physiology and structure is still too incomplete to permit following a purely rational approach to the design of a specific toxicant for a specific use. For some time progress will probably continue largely along empirical lines, but as our understanding of comparative physiology and biochemistry and of the subtle details of differences at the cellular level between species improves, we will move in the direction of a sounder scientific approach to control of plant diseases by chemical means.

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CHAPTER 14

Fungicidal Chemistry

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I. INTRODUCTION

This chapter will deal with the intrinsic properties of compounds that make them fungitoxic. The preceding chapter dealt with the biological pathways susceptible to poisoning.

As is the case of almost any discussion of chemical properties relating to biological action, there will be considerable simplification. Killing of living cells by a chemical compound is not a simple process, but rather the total observable response to a single or multiple chain reaction. Whether or not a specific property of a compound will result in the death of a specific cell or tissue very often depends on an exquisite interaction between the physical-chemical properties of the compound and those of the cell affected. For example, fluoroacetic acid is produced in the poisonous plant *Dichapetalum* without harm to the plant. Peters (1952) has found that when mammals ingest the plant, the fluoroacetate is mistaken for acetate in the Krebs cycle and fluoroacetate is produced which inhibits aconitase. This causes citrate to accumulate. Peters feels that the excess citrate is the toxicant. Here is a delicate change with a profound effect. Fluoroacetate is not a direct poison, but may be changed by mammalian tissue into another compound, fluorocitrate, which causes the accumulation of the toxicant. It would indeed be an oversimplification to ascribe to one property of fluoroacetate the ability to kill directly.

What must a compound be able to do in order to poison a cell? Simply stated, a poisonous compound must be able to get to a site of action, resist changes in transit, and exert a deleterious effect when it arrives. Requirements for poisonous molecules will, therefore, differ according to where they exert their effect. If they act outside the cell or at its surface, they will have no need to penetrate the intricate cellular membranes. In this case, the requirements for toxicants would be much simpler and less specific than for compounds which must penetrate.

Most organic molecules enter living cells most efficiently in the nonionized state. Organic toxicants, therefore, are usually most toxic in their nonionized form.

Are molecules always most toxic in their nonionized form? Albert (1951) and his group have found that the bactericidal effectiveness of the acridines and related series depends on their ability to ionize. Also, the addition of an aliphatic side chain, which usually aids permeation and attendant toxicity, actually reduces the bacteritoxicity of these compounds. Albert has deduced that acridine must act at the surface of the bacterial cells.

The 8-quinolinol derivatives, on the other hand, must be nonionized for maximum effect. If, as in the case of 8-quinolinols, the toxic molecule must enter the turbulent laboratory of the living cell, the molecule must resist destruction before it reaches its site of action. Rich and (Horsfall, 1954a) have demonstrated correlation between the detoxifying enzymes present in fungi and the resistance of these fungi to poisoning.

What does this mean in terms of comparative reactivity? A toxic molecule which is highly reactive and relatively unstable would be most likely to act in the outer portions of the cell. Great reactivity might serve to destroy and detoxify such a molecule before it moved very deeply into the cell. This property may, in fact, destroy a compound in the ambient fluid before it reaches the cell. For example, the presence of cysteine in the ambient fluid destroys captan before it can poison yeast cells (Lukens and Sisler, 1958). Lukens and Rich (unpublished) find that if yeast cells are treated with cobalt and then washed free of excess cobalt, they are more susceptible to captan than cells not treated with cobalt. Assuming that cobalt ties up surface sulfhydryl groups (Nickerson and van Rij, 1949), the cobalt pretreatment presumably prevents the partial breakdown of captan on its way to its site of action within the cell.

A more stable, relatively unreactive molecule would have a greater chance of penetrating unchanged to a site of action deeper within the cell. It will be seen that compounds considered chemically inert may be toxic.

Not only the relative site of the action, but also the mode of action dictates requirements for a toxic molecule. For example, a competitive antimetabolite has more and subtler requirements than those of a nonspecific poison, such as phenol. Sulfanilamide must not only be able to penetrate to a site of action and be resistant to detoxication, but in addition its molecular configuration must very closely approximate the metabolite *p*-aminobenzoic acid with which it competes within the cell. The distances between atoms in the molecule and their spatial relations are critical to the action of sulfanilamide.

Much has been said of the solubility of toxicants in relation to their toxicity. Does a compound have to be water-soluble to be toxic? How else can it be reactive in the water-flooded interior of the cell? Actually, such materials as copper oxide, sulfur, and dichlone, highly insoluble in water, are toxic. Very often water solubility is inversely related to toxicity as in the analogues of metallic 8-quinolinolates. This does not imply that good solubility in water precludes toxicity. The organic mercury salts, highly water-soluble, are excellent fungicides. Usually those toxicants which are water-soluble, are very reactive compounds exerting general nonspecific toxicity.

Why this wide variation in the relation between toxicity and water solubility? As the sites of action may be in polar or nonpolar milieu, or in a medium with both polar and nonpolar groups, e.g., proteins, toxic compounds must and do vary in their solubilities depending on where they must go to become toxic.

Most often in the case of organic toxicants, of primary importance is not water solubility or lipid (nonpolar) solubility, but rather the partitioning of the compound between the two phases. Rich and Horsfall (1952) state the case for the importance of this relationship in the action of fungicides. Working with a series of nitrosopyrazole analogues, they showed that increasing the oil-water partition coefficient from 0.48 to 6.66 increases fungitoxicity ten thousandfold with no appreciable change in the dosage response slopes of the analogues. They interpret this to mean that the change in the direction of high oil-water partition has greatly increased the penetration of the compound into the cell without altering the mode of action.

This solubility balance may be so critical that it may decide whether a compound will be toxic to one plant species and not to another. Wellman and McCallan (1946) working with 2-substituted imidazoline derivatives found that the tridecyl derivative was fourteen times more phytotoxic than the heptadecyl derivative. The heptadecyl homologue, however, was twice as toxic to fungi as the tridecyl homologue.

A more complicated relationship between toxicity and comparative solubilities exists in the 8-quinolinol derivatives. Albert and Hampton (1954) and Albert *et al.* (1954) showed that the antibacterial action of oxine derivatives is directly and closely correlated with oil-water partition. Albert *et al.* (1953), with bacteria, and Block (1956) and Sijpesteijn *et al.* (1957), with fungi, have demonstrated by various indirect biological methods that the toxic species of oxine and its derivative is the water-soluble half chelate. When the system is flooded with oxine well in excess of that needed for full chelation, there is no toxicity. Excess oxine would maintain the full chelate within the cell, preventing it from going to the half chelate. Thus, high oil-water partition is essential for movement of the molecule to a site of action, but, once there, the water soluble half chelate must be present for toxicity. This does not imply that water solubility per se is essential to the toxicity of the half chelate but merely that high oil-water partition is essential to movement, and once inside the cell this condition is not essential to toxicity.

The toxicity of another group of compounds appears to be completely correlated with oil-water partition, with no evidence that any other property is involved. These are the so-called inert, nonspecific toxicants which follow the Ferguson effect, to be discussed later.

The properties aiding direct permeation into a cell may not be the only ones of importance in moving a damaging number of toxic molecules to the site of action. Sussman (1954) showed that dormant ascospores of *Neurospora* adsorb ethylenediamine tetraacetic acid to their surface. The adsorption takes place even if the spores are first killed by boiling. If the ethylenediamine tetraacetic acid is removed from the spores before their dormancy is broken, there is no toxicity, but ethylenediamine tetraacetic acid is toxic if present when germination processes begin. Sussman interprets this to mean that the ethylenediamine tetraacetic acid cannot enter the dormant spore. Not until the permeability properties of the spores change by the onset of germination can the toxic molecules enter. Lowry *et al.* (1957) presented evidence that metallic cations such as Ag^+, Cu^{++}, and UO_2^{++}, follow this same course. Here, then, is an example of adsorptive forces concentrating a toxicant at the surface of a cell, ready to enter when the permeability properties of the cell change.

The work of Sussman and his colleagues should not be taken to mean that metallic ions enter only in the fashion they describe. Miller *et al.* (1953b) have shown that fungus spores take up silver and cerium extremely rapidly and greatly concentrate the toxicants in so doing. With silver, for example, as high as 2124 p.p.m. of spore weight may be taken up by *Monilinia fructicola* spores in the first 30 seconds of exposure to a maximum possible dose of 4160 p.p.m. Cerium entered *Aspergillus niger* spores at the rate of 7625 p.p.m. of spore weight in the first 30 seconds of exposure to a maximum possible dose of 10,000 p.p.m. Miller *et al.* (1953b) point out that if doses of this magnitude were the result of simple adsorption, then the spore surface would have to be covered with multimolecular layers of toxicant. This does not appear likely.

Owens and Miller (1957) later studied the distribution of cerium and silver toxicants in spores. These workers disintegrated toxicant-treated spores by sonic vibration, then fractionated the spore fragments by centrifugation. They found that only 4% of the total amount of cerium or silver taken up was associated with the cell walls. Owens and Miller feel that adsorption of cerium or silver on spore walls or plasma membranes plays little or no role in the movement of these toxic ions into the conidia of their two test organisms, *Neurospora sitophila* and *Aspergillus niger*.

Having the properties necessary to move to site of action without being detoxified, how may compounds poison? The next section of this chapter will deal with possible physical and chemical properties of the compounds which may be deleterious to living cells. The following two sections discuss specific inorganic and organic toxicants in detail. The last section will deal with fashioning fungicides and with the future of fungicides.

II. DESTRUCTIVE PROPERTIES OF FUNGITOXICANTS

Very few fungitoxic mechanisms are fully understood. Possession of one of the properties discussed below does not necessarily make the molecule toxic, nor does it explain why the molecule is toxic. Rather, this is a discussion of chemical and physical properties by which molecules may disrupt essential life processes.

A. *Direct Chemical Interactions*

Toxicants may undergo direct chemical interactions with vital constituents of the cell. For example, irreversible addition or substitution reactions with important enzymes or substrates would effectively poison a cell. Even reversible reactions will inhibit manifestations of life, and eventually cause death if a continuing supply of the toxicant is fed to the cell. These reversible reactions, inhibiting only as long as the toxicant is present, exemplify fungistasis or bacteriostasis.

Whether or not a toxic reaction is reversible, will depend largely on the type of bonds formed between toxicant and vital component. Albert (1951) summarizes bonds of importance in biology and gives their usual bond strengths. The *van der Waals' bond* is a weak bond (about 0.5 kilocalories) which may occur when any two atoms or two different molecules approach each other. Atoms must be able to approach each other very closely if they are to be firmly bound by van der Waals' bonds. Although a single one of these bonds is weak, there may be many of them between molecules of toxicants and vital constituents. The formation of many van der Waals' bonds is more likely to occur between molecules which can fit close together because of their molecular configurations.

The hydrogen bond, with a strength of 2 to 5 kilocalories, is fairly common in biology. In biological systems the hydrogen bond donors are usually the groups $\equiv N^+ - H$, $=N-H$, or $-O-H$. The hydrogen bond accepts oxygen or nitrogen atoms, or sometimes chloride ions.

The hydrogen bond is apparently made by resonance between two structures where hydrogen is attached to one or the other of the atoms which it joins. There is supposedly no actual oscillation, but the distribution of electrons is intermediate between the two forms [A——H B] and [A H——B] and is represented by [A——B]. The hydrogen bond is stable, because the energy of the mesomeric state is less than that of either of the other structures.

Ionic bonds, with a usual strength of about 5 kilocalories, form between oppositely charged ions. This is the bond of salt formation.

By far the strongest is the *covalent bond* with bond strengths of from 40 to 100 kilocalories. This bond, in which combining atoms each contribute one shared electron, makes for essentially irreversible combinations between toxic molecules and vital components. Such reactions as alkylation, acylation, or halogenation are most likely to cause irreversible poisoning. For example, Burchfield and Storrs (1956) suggest that 2,4-dichloro-6(*o*-chloroanilino)-*s*-triazine may be so effective as a fungitoxicant because it alkylates amino acids.

Factors other than bond strength are also important in toxicity by direct chemical interaction. One of these factors is reaction rate. Burchfield and Storrs (1957) studied the importance of reaction rate in the activity of toxicants. Specifically, they investigated the kinetics of reaction *in vitro* between toxicants and vital constituents of cells. Although two toxicants may possibly react with the same group of a vital constituent, the ease and speed with which the reaction occurs may well decide whether the reaction is important to the toxicity of one compound and not the other. Some of the factors discussed were energies of activation, frequency factors, degree of solvation of a reactive halogen, and the degree of dissociation of the substrate. 1-Fluoro-2,4-dinitrobenzene was found to be a more highly specific reagent for sulfhydryl groups than is 2,4-dichloro-6(*o*-chloroanilino)-*s*-triazine. Conversely, the *s*-triazine is highly reactive with organic amines and phenols, while the 1-fluoro-2,4-dinitrobenzene is not. This latter difference is apparently related to the low dissociation constants of organic amines and phenols important to the growth of the organism.

Lukens and Sisler (1958) suggest that the toxicity of captan is related to double decomposition reaction of captan with sulfhydryl containing components of the cell. Here again, whether or not a compound will react in a certain way is not enough to make it a toxicant. The rate of reaction must be sufficiently fast to be effective. For example, if captan is toxic because of the reaction of $—S—C(Cl)_3$ with essential sulfhydryl groups, then the rest of the molecule may decide the toxicity of the compound as a whole. The phthalimide moiety of captan has carbonyl groups on each side of the N. These electron attracting groups weaken the N—S bond. If these electronegative groups were not present, or not in conjugation with the N, the resulting N—S linkage would be much more stable. The reaction rates would be slowed down and toxicity would be lessened. For example, our data show that *N*-trichloromethylthiocarbazole is much more stable than captan and also practically nonfungitoxic.

The heavy metals, such as silver and mercury, combine readily and firmly with sulfhydryl groups. Without question, this property contributes to their toxicity. Certain well-known specific reagents for sulfhydryl groups, such as iodoacetamide, are also toxic.

Not only are metals toxic because they may combine readily and firmly with cellular constituents, but also other molecules may be toxic because they combine with metals which are necessary to life. This latter mechanism has been proposed for chelating agents (Zentmyer, 1943). Binding metals is presumably the action of azide and cyanide.

Bisulfite, toxic to many organisms, is known to combine with pyruvate. This combination would inhibit the decarboxylation of pyruvate, essential to organic acid metabolism.

The α, β-unsaturated ketones, including quinones, form addition products with thiols and amino compounds. This property has been suggested (Geiger and Conn, 1945) as a basis for toxicity.

There are, then, a number of direct reactions between foreign molecules and cellular components which may lead to toxicity.

B. *Effects on Enzyme Systems*

One of the most popular areas of research on toxicants has been the study of their effects on enzyme systems. The primary reason for this has been the need to explain the profound effects produced by the application of extremely low dosages of toxicants. A most reasonable explanation is the assumption that the toxicants hit the organisms in a vital link in their metabolic chain. The many enzymes needed by organisms to change raw food materials into energy and living substance loom large as potential targets. These organic catalysts are not only themselves susceptible to inactivation, but also the processes they perform may be so altered as to result in toxicity. When these effects are described, therefore, they are presented in terms of effects on enzyme systems.

1. *Enzyme Inhibition*

The three principal kinds of inhibition of enzyme systems are termed competitive, noncompetitive, and uncompetitive.

Competitive inhibitors presumably poison by so closely resembling vital substrate that they are taken up by the enzymes which normally work on these substrates. The inhibitor molecules, however, differ sufficiently from the normal substrate molecules so that they cannot be changed by the enzymes into nutrilites needed by the cell to survive. The insertion of these ill-fitting keys into the active sites on the enzyme exclude the required substrate. The competition between the essential molecules and the imposter molecules for enzyme sites is in proportion to the ratio of the two competing molecules in the system. In other words, the degree of enzyme inhibition is proportional to the ratio of substrate to inhibitor. The more substrate, the more inhibitor required for the same degree of inhibition, and vice versa.

The classic Lineweaver-Burk (1934) plots of enzyme kinetics compare the reciprocal of substrate concentration S to the reciprocal of the observed reaction rate v. Figure 1 represents a typical plot for a case of competitive inhibition.

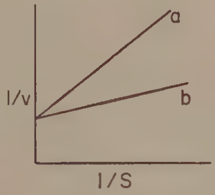


FIG. 1. Lineweaver-Burk plot for competitive inhibition.

The slope V of the inhibited line (a) is much steeper than the slope of the noninhibited line (b). The intercepts with the ordinate, however, are identical.

Equation 1 describes the steady state for competitive inhibition. At steady state $V =$ maximum velocity when the enzyme-substrate combination is maximum.

$$v = \frac{V}{I + K_s[I + (I)/K_i]/S} \qquad \text{(Eq. 1)}$$

$K_s =$ Michaelis constant, which is the substrate concentration at which half maximum velocity is reached.

$I =$ Concentration of inhibitor.

$S =$ Concentration of substrate.

$K_i =$ Constant for dissociation of enzyme-inhibitor combination into enzyme and inhibitor.

As S is increased it affects both K_s and V. When S is sufficiently large, inhibition is completely overcome, and the intercept at sufficient value of S becomes identical with that of no inhibitor. The slopes of the two systems, one with and the other without inhibitor, differ by the function $[I + (I) / K_i]$.

Noncompetitive inhibition applies to the case where the inhibitor combines with the enzyme at a point other than the site at which the substrate attaches. There are two other requirements for this type of inhibition: (a) the inhibitor exerts a total inhibitory effect; and (b) the binding of inhibitor to enzyme is independent of substrate. Under these conditions, the Lineweaver-Burk plot shows lines differing in both slope and intercept (Fig. 2).

Equation 2 describes such a system.

$$v = \frac{V}{[I + K_s/S][I + (I)/K_i]} \qquad \text{(Eq. 2)}$$

Here, as in the preceding equation, the slope of V is still influenced by the inhibitor function, so that the slope of the inhibited system varies from the slope of the noninhibited system by $[I + (I) / K_i]$. It differs, however, by not having the inhibitor function modified by substrate concentration. Hence, at the intercept, the lines are separated by $[I + (I)/K_i]$. In other words, the reaction rate of a system noncompetitively inhibited can never attain the rate of the noninhibited system, regardless of the amount of substrate added.

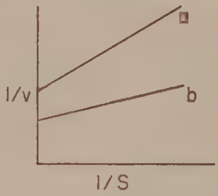


Fig. 2. Lineweaver-Burk plot for noncompetitive inhibition.

Uncompetitive inhibition requires that the inhibitor combine only with the enzyme-substrate complex. In these circumstances, the Lineweaver-Burk plot shows parallel slopes for the two systems, and of necessity, different intercepts. Equation 3 describes uncompetitive inhibition.

$$v = \frac{V/[I + (I)/K_i]}{I + K_s[I + (I)/K_i]/S} \qquad \text{(Eq. 3)}$$

Other special cases include inhibition by a partial inhibitor, and inhibition by one of the products of the normal substrate with enzyme.

What does all this mean in terms of *in vivo* toxic effects? The great interest in competitive inhibitors as a toxic mechanism stems from the classic research of Woods (1940), who demonstrated that sulfanilamide is a competitive inhibitor of *p*-aminobenzoic acid. Not until after Woods' report was *p*-aminobenzoic acid found to be an essential metabolite. Since that time, whenever a toxicant is found which bears a structural resemblance to a vital substrate, competitive inhibition is invoked.

Actually, proven cases of competitive inhibition as a fungitoxic mechanism are not too common. West and Wolf (1955) present strong evidence that 2-heptadecyl-2-imidazoline is a competitive antagonist for guanine and xanthine in *Sclerotinia fructicola.*

Because a compound may act as a competitive inhibitor on one organism is no reason to believe that it acts as such in another. Usually, competitive inhibition is most striking in those cases where the organism is heterotrophic for the particular metabolite being antagonized.

A very striking example of the complexity of enzyme inhibition as a toxic mechanism is described by Woolley and Shaw (1951). They found that 2-azaadenine is particularly lethal to *Lactobacillus* species.

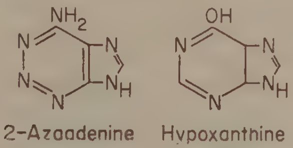


FIG. 3. Hypoxanthine (right) and its antimetabolite, 2-Azaadenine (left).

They tested the purines, hypoxanthine, adenine, and xanthine as possible metabolites being antagonized, with the following results. 2-Azaadenine competitively inhibits hypoxanthine (Fig. 3). Its toxicity is largely overcome by trace amounts of adenine, but is not appreciably alleviated by large amounts of xanthine. Without the inhibitors, xanthine is as effective as hypoxanthine in the purine nutrition of these bacteria. From their results, it becomes obvious that 2-azaadenine is highly specific in its effects on *Lactobacilli.* It is not simply a competitive inhibitor of purines as a class, but of one purine in particular, hypoxanthine.

Their experiment again serves to illustrate the physiological implications that may result from toxicological studies. Their results indicate that the chain of nucleoside synthesis in *Lactobacillus* goes from xanthine → hypoxanthine → adenine. If 2-azaadenine breaks the chain at hypoxanthine, the addition of more xanthine could have little effect. The addition of adenine, however, would be beyond the block and nucleic acid synthesis could continue.

Noncompetitive inhibition as a toxic mechanism may be postulated for almost any toxicant which can denature proteins. For example, picric acid, a protein precipitant, is quite fungitoxic and may act by denaturing proteins. The same may be said for formaldehyde. Any of the amino acid reagents shown to be fungitoxic by Horsfall and Zentmyer (1944) may be acting as noncompetitive inhibitors.

Uncompetitive inhibition has not been postulated as a toxic mechanism for any one particular known toxicant. This type of inhibition must surely contribute to the toxicity of some poisons.

Many known fungitoxicants probably act as nonspecific enzyme inhibitors. The enzyme proteins may lose their activity if the attacking toxic molecule has a particular avidity for, or ability to react with any of the reactive groups on the protein.

The susceptibility of enzymes may be realized when we consider the number of reactive sites possessed by amino acids which make up the proteins. Some of these reactive sites are primary amino groups ($—NH_2$), alcohol groups intermediate between aliphatic and aromatic (the —OH of hydroxyproline), the imino group (=NH), the acid amide group ($—CONH_2$), the sulfhydryl group (—SH), the α-hydrogen of tryptophan, and the guanidine nucleus. The —OH groups of hydroxyamino acids may be of particular importance as susceptible sites because they combine with phosphoric acid to bridge polypeptide chains.

There are other toxic mechanisms closely related to enzyme inhibition which may well be discussed here. One of these, a variation on the theme of competitive inhibition, is a phenomenon classified by Markham (1958) as a type of "lethal synthesis." In this particular mechanism, the imposter molecules are so like the normal substrate molecules that the toxicants move along the chain of synthesis and are actually incorporated into the final building blocks of living substance. The building blocks containing these imposter molecules are defective and cannot be utilized in a normal fashion by the organism. The organism shows toxic effects as a consequence.

Heinrich *et al.* (1952) found that the protozoan *Tetrahymena geleii*, which is readily poisoned by 8-azaguanine, incorporates this aminopurine into its ribonucleic acid when grown on sublethal doses of the toxicant. The toxic effect of 8-azaguanine is readily overcome by the simultaneous addition of the nucleoside, guanosine.

Another example of this type is reported by Dunn and Smith (1957). They found that *Escherichia coli* made sensitive to poisoning by 5-bromouracil incorporates this halogenated pyrimidine into its deoxyribonucleic acid. The toxic effect of this compound cannot be overcome by the addition of thymine for which it is known to be an antimetabolite. *E. coli* is not inhibited by 5-bromouracil nor does it incorporate it unless the bacteria are made to require thymine by treatment with sulfanilamide.

To call this type of toxicity a form of competitive inhibition may not be strictly proper, as it may or may not be reversible. It is quite apparent, however, that in these cases there must be competition between normal substrate and imposter. Once the imposter molecules become incorporated into the nucleic acids the toxic molecules may or may not be replaceable.

2. *Enzyme Stimulation*

Not only may toxicants act by inhibiting enzymes, but they may also act by overstimulating enzymes. Such overstimulation may throw the normal cellular physiology completely out of balance. This abnormal unbalance may be analogous to an overdose of insulin in humans. Byrde *et al.* (1956), with *in vitro* studies of enzymes from *Monilinia laxa*, showed that copper greatly stimulates the activity of DPN oxidase, while *o*-phenylphenol greatly stimulates cytochrome c oxidase. Mahler *et al.* (1955) suggest a plan to explain the burst of molecular oxygen released from systems containing metalloflavoprotein enzymes (EF-Fe) when they are acted upon by quinones. Normally the electron transfer goes DPNH → EF-Fe → Cytochrome c. With quinone in the system, the electrons may be diverted completely from EF-Fe to molecular oxygen rather than cytochrome c, thus uncoupling all subsequent phosphorylations.

C. *Chelation*

Before presenting chelation as a toxic mechanism it would be well to know something about chelates.

Chelates are cyclic structures formed by the union of metallic atoms with inorganic or organic molecules or ions. The term comes from *chela*, the Greek name for crab's claw. One molecule of the nonmetallic portion of the complex is called a ligand. Compounds which chelate must be able to combine at more than one point with metals. A simple 2 point, or bidentate, ligand is pictured in Fig. 4. Each 8-quinolinol molecule is a ligand.

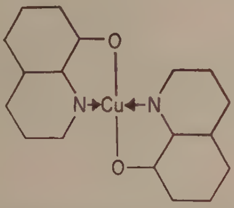


FIG. 4. 8-Quinolinol combining with copper. An example of bidentate ligands.

Note that the metal is combined with each ligand by two bonds: (a) an ionic bond, and (b) a coordinate bond. In a coordinate bond, both of the electrons shared by the combining atoms come from only one of the atoms, nitrogen in this case. Other chelates are known in which all the bonds between ligands and metal are coordinate, without any ionic bonding.

It is the multiple bonding feature of chelation which gives chelators their most interesting property. This property is the formation of an extremely stable complex which can hold metals so tightly that they are released or exchanged only with great difficulty.

The formation of chelate compounds is usually accompanied by color formation and the loss of polar properties by the ligand molecules. As the atoms on the ligand which combine with metals are usually nitrogen, oxygen, or sulfur, it is quite apparent why polar properties are lost. Whether or not the complex remains water-soluble depends on whether or not the ligand has polar groups not involved in chelation. For example, copper 8-quinolinolate is quite insoluble in water, while copper 8-quinolinol-5-sulfonate is water-soluble.

The smaller chelated cyclic structures are considered to be coplanar. According to the Baeyer strain theory, these, like other organic cyclic compounds, would be most stable when there are 5 or 6 atoms in each ring. Chelate cycles with less than 4 atoms or more than 7 atoms are known, but they are uncommon and usually unstable. Because of certain peculiarities of chelate rings, 4-membered structures are more common and more stable than 4-membered carbon rings. The probability of a compound being a chelator is highest if it has 2 electron-sharing atoms separated by 2 or 3 other atoms.

In the union of ligand with metal, the ligand is considered to be the electron-pair donor, or base, while the metal is the acceptor, or acid. All other things being equal, the greater the basic strength of the ligand, the more stable the chelate complexes it can form.

What properties of the metal influence the stability of the complex? Irving and Williams (1953) present the following three features as being the most important metallic properties in chelation: (a) valence, (b) the ionic radius, and (c) the degree of ionization.

The higher the cationic valence the more stable the complex. For example, Fe^{+++} complexes are much more stable than Fe^{++} complexes.

The larger the ionic radius the less stable the complex. Mn^{++} has a much larger ionic radius than Cu^{++} and usually forms less stable chelates.

Other things being equal, chelate stability is directly correlated with the second ionization potentials of the metals.

Chelating compounds are both common and important in biology. Among them are organic acids, amino acids, enzymes, vitamins, hemoglobin, and chlorophyll.

Chelation as a toxic mechanism has been a subject of active research and debate since it was first proposed by Zentmyer for fungi (1943) and by Albert for bacteria (1944).

Zentmyer (1944) showed that the toxicity of the powerful chelator

8-quinolinol (oxine) to the fungus can be overcome by the addition of excess zinc to the growth medium. Furthermore, reducing the pH of the medium to an acidity at which oxine cannot chelate, also destroyed its fungitoxicity. On this evidence, Zentmyer related the fungitoxicity of oxine to its ability to chelate, and proposed that oxine poisons by robbing the organism of required trace metals. Albert and his group were also able to destroy the antibacterial action of oxine by the addition of excess metal, iron in this case. In addition, they found that isomers or analogues of oxine able to chelate, were also antibacterial. Those related compounds unable to chelate, e.g., 2-quinolinol, were not antibacterial.

Soon, however, the metal-robbing theory was seriously questioned by Mason (1948) who showed that copper oxinate is more fungitoxic than the unchelated oxine. This was particularly damaging to the theory of metal-robbing. Since the copper chelate is one of the most stable of oxinates, it would not be expected to chelate other trace metals. Albert and his group found that oxine is not antibacterial unless iron is present in trace amounts. It must be made clear here that chelation as a toxic mechanism was not questioned. In doubt was only the theory that the toxicity of oxine derives from metal-robbing. It is ironic that the metal-robbing theory of toxicity should have been based on oxine which may or may not work in that way. There is not much doubt that chelators may exert their toxicity by metal-robbing. For example, Rich and Horsfall (1948) have shown that dimethylglyoxime will produce trace metal deficiency symptoms in *Aspergillus niger*. On dimethylglyoxime, the fungus grows in curds, rather than in a normal mat, and sporulation is poor or absent.

In what other way may chelators or chelate compounds poison? Albert and others have suggested that the poisonous form of oxine is the half chelate. Goksøyr (1955) also proposes the half chelate as the toxic species of dithiocarbamates. If this is so, then the half chelates may be poisoning by blocking functional groups. For example, an amino group and hydroxyl group properly spaced on the surface of an enzyme protein would form a mixed chelate with the half chelated toxicants. This is illustrated in Fig. 5.

The same effect could be had with a sulfhydryl group in place of the amino or hydroxyl group.

Another type of functional group blocking would be the formation of mixed chelates with the phosphoric acid or nitrogen base portions of nucleotides. This may be illustrated (Fig. 6) with copper oxinate and adenosine-5′-diphosphate (ADP).

So much for chelators and their possible toxic mechanisms. The

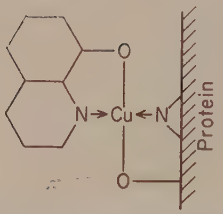


FIG. 5. Half chelate blocking functional groups.

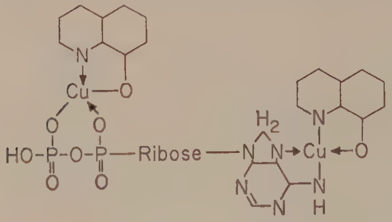


FIG. 6. Half chelate blocking ADP.

fascinating stories of oxine and dithiocarbamates as toxicants will be described in detail in a later section.

D. *Selective Solubility*

Toxic compounds may act by selective solubility. Ferguson (1939) has shown that the insecticidal value of many chemically "inert" materials, such as saturated hydrocarbons, is correlated with their relative solubility in the medium in which they are applied. He pointed out that the partition of these compounds between the ambient medium and the biophase will be proportional to the degree of saturation of the ambient medium. In other words, the less soluble the toxicant in the applying medium, the greater the saturation of that medium and the greater the proportion entering the biophase. The degree of toxicity of these structurally nonspecific compounds is directly proportional to their saturation of the medium in which they are applied. For example, nonspecific compounds X, Y, and Z may require respectively 1000 p.p.m., 100 p.p.m., and 1 p.p.m. to half-saturate the medium in which they are applied, whether it be water or air. These dosages should approximate half the maximum toxicity possible for treating organisms with compounds X, Y, and Z.

Ferguson was able to use this effect to explain another situation common in toxicity studies. Very often in comparing homologues of a toxic molecule, toxicity increases as the alkyl side chain lengthens. This continues until the addition of one more methyl group makes that homologue almost nontoxic. Ferguson suggests that the final methyl group would make the compound so insoluble in the applying fluid, that the amount required for complete saturation would partition less than a toxic dose into the biophase. This cut-off point will vary with compounds, applying medium, and tissue poisoned.

Ross and Ludwig (1957) have presented evidence that the fungitoxicity of the *N-n*-alkylethylenethioureas follows the Ferguson effect.

All this is interesting and useful, but does not explain how supposedly "inert, non-specific" compounds can poison. There are at least two possibilities.

The first is a solvent effect. If the inert molecules partially replace and force apart the biophase molecules, metabolic reaction rates could be altered enormously. Taking a test-tube example from organic chemistry, the reaction of ethyl iodide with triethylamine is only 1/1000 times as fast in hexane as it is in benzyl alcohol. The nonspecific toxicants could slow down vital reaction rates by reducing the frequency with which substrate molecules must collide with enzyme molecules essential for continuing normal processes.

The second way in which nonspecific toxicants may act is by altering the colloidal structure of the semipermeable membranes of the cell. Many of these toxicants have a particular avidity for the lipoproteins of the cellular membranes. They are also known to exert cellular narcosis, allowing cellular contents to leak from the cell. The most likely way that these compounds could do this is by affecting the structure of the hydrophilic colloids making up the semipermeable membranes.

E. *Dissolution*

Toxicants may act by dissolving cellular components necessary to the physical structure of the cell. A good example is lysozyme, an antibiotic enzyme discovered by Fleming (1922) in mucous secretions. Lysozyme is a mucopolysaccharide depolymerase. It is capable of completely dissolving the cell walls of bacteria. Commercial lysozyme, prepared from egg whites, is assayed by its ability to dissolve suspensions of the bacterium *Micrococcus lysodeikticus*. The end-product of lysozyme activity is *N*-acetylglucosamine. There is no increase in reducing groups. Lysozyme has been found in many bacteria, including those susceptible to its activity. It also occurs in *Ficus latex* and in commercial papain.

Rich (unpublished) has found that lysozyme, with no apparent effect on spore walls, will lyse the germ tubes of *Stemphylium sarcinaeforme*, allowing the cellular contents to escape. Rich also found that sodium hypochlorite causes the separation of the cells of this multicelled spore.

Carter and Lockwood (1957) described the lysis of the mycelium of *Glomerella cingulata* by a diffusate from *Streptomyces* sp. They also report the lysis of *G. cingulata* mycelium by antibiotics such as mycostatin and thiolutin, and by fungicides such as ferbam, thiram, and zineb. The latter effect they ascribe to a hastening of autolysis of the poisoned mycelium, rather than a direct dissolution of the mycelium by the fungicide.

A material may not cause a complete dissolution of cell wall, but may instead have a partial effect on its rigidity. Griseofulvin may act in this fashion. This antibiotic was found to be the "curling factor" produced by *Penicillium janczewskii* (Brian *et al.*, 1946). It causes a spiral growth of the germ tubes of *Botrytis allii* and other fungi. This abnormality of the germ tubes apparently prevents the organism from developing mycelium.

F. *Precipitation*

Precipitation, the opposite of dissolution, may poison either by making metabolites unavailable or by destroying colloidal structure.

Polysulfides, present in lime sulfur, may produce their poisonous effect by precipitating essential metals and so making them unavailable. *Aspergillus niger*, grown in the presence of potassium sulfide, will produce yellow, rather than black, spores. This is a symptom of metal deficiency. Hydroxides or carbonates may also produce this effect.

Precipitation or coagulation of hydrophobic colloids, such as proteins, would also have a profound effect on normal cellular processes. The ability of a compound to bring about coagulation will depend on two things. First, the power to neutralize the charge on the dispersed phase, or the lowering of the zeta potential; and, second, the ability of the coagulant to take the stabilizing hydration water from the dispersed particles.

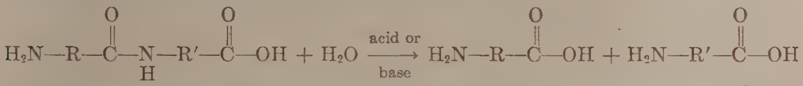
The ability to neutralize charges will depend on the valency of the ion charge oppositely to that of the dispersed phase. For example, if a protein sol is at a pH on the alkaline side of its isoelectric point, the dispersed phase will be negatively charged. The ability of the coagulant to reduce the charge on the protein micelle will be directly related to the valency of the cation of the coagulant. Actually, very small amounts of oppositely charged ions may cause a large drop in the zeta potential. The hydrophilic colloid may increase in viscosity as its charge is neutralized, but coagulation is prevented by the stabilizing effect of the hydrated shells around each micelle.

The final phase of coagulation depends on the ability of the toxicant to dehydrate. This ability to take water from the particles usually follows the lyotropic series. For the effect of anions to precipitate proteins, this series is usually shown as follows: citrate > tartrate > SO_4 > acetate > Cl > NO_3 > Br > I > CNS. Citrate shows the greatest effect and thiocyanate the least. This series for cations is, Th > Al > H > Sr > Ca > K > Na > Li. Ethanol and formaldehyde are two other toxicants which may act as protein coagulants.

G. *Hydrolysis*

Acids or bases may poison by hydrolysis. Hydrolysis is the breaking of the reacting molecule and the combination of the pieces with H^+ and OH^- from water.

From the viewpoint of toxicity, proteins are probably the most important biological compounds which may be hydrolyzed. Hydrolysis would, of course, be a denaturing process, particularly disruptive to enzymes. When enzymes are hydrolyzed, the most profound effect is the breaking of peptide linkages which join amino acids to make the peptide chains of which proteins are built (Equation 4).



$$H_2N{-}R{-}\overset{\overset{O}{\|}}{C}{-}\underset{H}{N}{-}R'{-}\overset{\overset{O}{\|}}{C}{-}OH + H_2O \xrightarrow[\text{base}]{\text{acid or}} H_2N{-}R{-}\overset{\overset{O}{\|}}{C}{-}OH + H_2N{-}R'{-}\overset{\overset{O}{\|}}{C}{-}OH \qquad \text{(Eq. 4)}$$

Mandels (1953) used weak acid treatment to destroy the activity of enzymes presumably located at or near the surface of *Myrothecium* spores.

Blood (1937) suggested that the acid production by fermenting tomato pulp is responsible for the elimination of *Corynebacterium michiganense* from tomato seed. He showed that soaking infested seed in acetic or lactic acid does as well as the fermentation process.

Tyner (1951) made the surprising discovery that merely soaking barley seed in water for a period of time can control loose smut. An investigation of this phenomenon by Leben *et al.* (1956), and Jacquet *et al.* (1957) showed that the control is achieved by organic acids. These organic acids, acetic, butyric, and formic, produced by bacteria operating during the water soaking, are the actual toxicants.

H. *Oxidation-Reduction*

Oxidation-reduction changes, in order to cause any real damage to cells, must be drastic rather than mild. Living cells usually have too large a capacity to equilibrate changes in oxidation-reduction to expect any lasting toxicity from mild changes. Calcium hypochlorite, a strong oxidant, is very toxic. Whether or not it acts on a specific system is not important. It is probably nonspecific by virtue of its capacity to oxidize strongly many vital components within the cell. Ozone, another strong oxidant, is toxic to many types of cells. Its production by ultraviolet radiation may account in part for the deleterious effects of UV light. Such systems as $Cu^{++} \rightleftharpoons Cu^{+}$ may be poisonous by virtue of their oxidant action. For example, Albert (1952) reported that Cu^{++} reacts rapidly with cysteine to give cystine and cuprous cysteinate. Other toxic oxidizing agents are SO_2, H_2O_2, potassium permanganate, sodium perborate, and sodium dichromate. As will be discussed later, elemental sulfur may poison by acting as an oxidant.

Horsfall (1945) suggested that the dinitrophenols are toxic because they can oxidize and so split unsaturated fatty acids. Dinitrophenols are very effective against powdery mildews. Horsfall later (1956) proposed that these compounds kill conidia of powdery mildew by destroying the fatty envelope that retains the water these spores need to survive. The spores then die by desiccation.

Hydrogen sulfide is a powerful reducing agent particularly toxic to cells. It may act not only by precipitating metals, but also by holding them in a reduced state.

Oxidation-reduction systems may show definite antibacterial action not shown by either component of the system. Guest and Salle (1942) found that a combination of $Fe\ Cl_2$ with either $Fe\ Cl_3$ or $Fe_2\ (SO_4)_3$ is much more antibacterial than any of these compounds used separately. This was also true of $Sn\ Cl_2$ with $Sn\ Cl_4$, $Fe\ Cl_2$ with $Sn\ Cl_4$, $Sn\ Cl_2$ with $Fe\ Cl_3$, $Mn\ SO_4$ with $Sn\ Cl_4$, and $Mn\ SO_4$ with $Fe_2\ (SO_4)_3$. These heavy metal salts were most toxic when mixed in certain proportions. The increase in toxicity required that the mixture had one metal at a higher oxidation state than the other. Only the salts of heavy metals gave this effect.

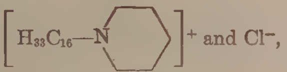
I. *Surface Activity*

Surface activity is a property of any substance whose presence in small amounts greatly alters the surface behavior of a given system. "Greatly alters" is rather a loose term, as most solutes will alter the surface behavior of the solvent to some extent. Although there are water-insoluble surfactants, we will be concerned with water-soluble organic compounds that markedly lower the surface tension of the liquid system in which they are dissolved. The most common of these surfactants is, of course, soap. A burgeoning chemical industry has produced a great number of synthetic surfactants that have found uses in all phases of our daily life.

What are the molecular properties that make a surfactant? Basically, a surfactant molecule is a long molecule, one end polar and the other end nonpolar. The polar end has an affinity for polar substances, such as water, while the nonpolar end has an affinity for nonpolar substances, such as grease, fats, and oils. When surfactant-containing water is applied to grease particles, the nonpolar end of the surfactant molecules is adsorbed by the grease. Since the polar end will not dissolve in the grease, the surfactant molecules stay at the surface. The surface-located polar ends having an affinity for water give the grease particle a miscibility with water. This allows the grease particles to become suspended in water, or emulsified, and so removed.

Surfactants lower the surface tension of water because the nonpolar ends of the surfactant molecules are forced out of water, and are concentrated and arranged linearly at right angles to the surface of the liquid, the polar end toward the liquid and the nonpolar end forced away from the liquid. The surface then acquires primarily the nonpolar properties of the surfactant. As the attraction between surfactant molecules is much less than the attraction of one water molecule for another water molecule, the surface tension is lowered. The reason why only a small amount of surfactant is required is that it is forced to the liquid-air or liquid-solid interface and all that is required for maximum effect is a unimolecular layer of the surfactant molecules at the interface.

There are three kinds of surfactants, anionic, cationic, and nonionic. The ionic surfactants ionize or dissociate into two parts, a large fatty radical and a small salt radical. If the charge in the fatty radical is negative, the compound is anionic. For example, a soap dissociates into $C_{17}H_{35}COO^-$ and Na^+, typical of an anionic surfactant. If the charge on the large radical is positive, the compound is classed as cationic. Cetylpyridinium chloride ionizes into



making it a cationic surfactant. Nonionic, or ampholytic surfactants form zwitterions in solution. An example is cetylaminoacetic acid: $H_{33}C_{16}NH^+—COO^-$.

Soap is a weak germicide. Its primary value as a sanitizer is to remove germs physically rather than to kill them. The real interest in the toxic and germicidal effects of surfactants dates from the work of Domagk (1935) who found that some cationic surfactants are powerful antibacterial compounds. Since then a number of quaternary ammonium compounds have been developed both as bactericides and fungicides. These have been principally alkylpyridinium halides and alkyl trimethylammonium halides. These cationic compounds are effective against both gram-positive and gram-negative bacteria and against fungi. Schwartz *et al.* (1958) list 43 different types of cationic surfactant germicides.

Anionic surfactants are primarily active against gram-positive bacteria. Examples of anionic surfactant toxicants are soaps and certain aliphatic carboxylic acids.

Nonionic surfactants usually have little or no germicidal action. Schwartz *et al.* (1958) list the salts of dioctylaminoethylglycine as examples of nonionic germicidal surfactants.

Surfactants appear to exert their toxic effects by breaking cross-links between polypeptide chains, or by disrupting colloidal structures. The effect on polypeptide structures is evident in the report of Sreenivasaya and Pirie (1938) who found that the protein of tobacco mosaic virus is disintegrated by surfactants. Anson (1939) also found that surfactants could split proteins into much smaller molecular units.

The profound effect of surfactants on colloidal structure is noted in toxicity studies by a disruption of the semipermeable membranes allowing the leaking materials from the cell. Horsfall (1956) discusses the importance of this as a toxic mechanism in both bacteria and fungi. Salton *et al.* (1951) have taken electron micrographs of bacteria treated with cetyltrimethylammonium bromide and have observed morphological changes correlated with cell death. Bacteria treated with 45 mg. of cetyltrimethylammonium bromide per 1.5 mg. dry weight of bacteria for 5 minutes show a pulling away of the cytoplasmic membrane from the cell wall. This dosage of cetyltrimethylammonium bromide just initiates leaking. The proportion of cells (30%) killed by this treatment is the same as the number of treated cells showing contracted cytoplasm. Treating the cells with 900 mg. cetyltrimethylammonium bromide per 1.5 mg. of bacteria for 30 minutes strips off the cell walls.

J. *Osmotic Pressure Changes*

Probably the oldest means of killing microorganisms is the preservation of foods with compounds that produce high osmotic pressures. Salting and sugaring are ancient means of preventing the growth of molds and bacteria on foods. Although the effective dosages of these materials are very high, salt and sugar were usually close at hand. On a dosage basis, this toxic mechanism is very inefficient, and will probably not be of importance in the development of future fungicides.

There are probably other toxic mechanisms of importance, but those listed here undoubtedly include those of principal interest.

It is entirely possible for a single toxicant to have more than one mode of action, one of which will be effective on one organism and another mode on a second organism. One can picture highly reactive molecules, such as captan, passing into the cell and reacting with metabolites *en route*. Toxicity or death of the cell results when any vital link is broken.

III. INORGANIC FUNGITOXICANTS

A. *Metals*

The most widely used inorganic toxicants are metallic compounds. Of the metals used as fungitoxicants, copper, specifically Bordeaux mixture, has been used in the largest amounts. Actually, in terms of minimum toxic dosages, inorganic metallic compounds are not nearly so toxic as a multitude of organic, nonmetallic toxicants. Even the organic metal complexes are more potent than the inorganic compounds containing the same metals.

According to Horsfall (1956), the descending order of fungitoxicity for the metal cations is approximately $Ag > Hg > Cu > Cd > Cr > Ni > Pb > Co > Zn > Fe > Ca$. McCallan and Wilcoxon (1934) found also that osmium and ruthenium are quite fungitoxic. Aluminum and palladium are only slightly toxic, and rhodium and iridium are essentially nonfungitoxic. McCallan and Wilcoxon (1934) studied the fungitoxicity of the elements in relation to their position on the periodic table. They generalized that toxicity within a group usually increased with increasing atomic weight. The principal exceptions are silver, much more toxic than gold, and chromium, usually more toxic than molybdenum and tungsten. A study of the periodic table shows that most toxic metals fall in groups I, II, and VIII.

Horsfall (1956) pointed out the good correlation between the toxicity of metals and their ability to form stable chelate compounds. His comparison of chelate stability (Mellor and Maley, 1948) with the toxicity of the metals is given below.

Toxicity: $Hg > Cu > Cd > Ni > Pb > Co > Zn > Fe > Ca$.
Chelate stability: $Hg > Cu > Ni > Pb > Co = Zn > Cd > Fe > Mg$.

Horsfall regards this as evidence that the primary toxic mechanisms of metals is their ability to combine with metabolites, enzymes, and proteins. Palladium and silver are two glaring exceptions to Horsfall's generalization. Palladium, only weakly toxic, forms very stable chelates, while silver, highly toxic, forms only weak chelates.

Shaw (1954) correlated toxicity with the insolubility of the metal sulfide. His diagrams show excellent correlations between the negative logarithm of lethal dose and the solubility product constants of the various metallic sulfides. This correlation was fairly consistent for the poisoning of diastase, urease, the paramecium, a planarium *(Polycelis* *nigra),* or a fish (the stickleback). Shaw believes this is the result of the "thermodynamic affinity of the inhibitor [metallic cations] for key functional group in the enzyme's catalytically active site [sulfhydryl groups]."

Shaw, of course, was preceded by Fildes (1940) who showed that mercury toxicity could be alleviated by sulfhydryl-containing compounds. Fildes suggested that mercury at low concentration acts by inactivating sulfhydryl groups without other permanent injury to the cell. British anti-Lewisite (2,4-dimercaptopropanol) was developed specifically as an antidote for the arsenical war-gas Lewisite ($Cl\,CH = CH.As\,Cl_2$). The development of BAL was based on the discovery that arsenic poisons by knocking out sulfhydryl groups.

Sulfhydryl compounds will also reverse copper toxicity of peas (Foster, 1947) and of fungus spores (Yoder, 1951). Foster showed that three kinds of seeds susceptible to copper injury contain sulfhydryl groups detectable with nitroprusside. Four kinds of seeds tolerant to copper contain no detectable sulfhydryl groups. Copper seed-treatments eliminated the detectable sulfhydryl groups of the copper-sensitive seeds. Both Yoder and Foster concluded that the sulfhydryl groups poisoned were essential to the functioning of the respiratory enzymes.

Metallic compounds are often used as astringents in medicine. Astringency is the precipitation of proteins by high concentrations of metallic compounds.

An obvious question is the relation of toxicity to the oxidation state of the metal. Apparently no generalization can be made concerning this point. Cupric ions are fungitoxic, but cuprous oxide is more toxic than cupric oxide (Horsfall *et al.*, 1937). Mercuric ions are usually more toxic than mercurous ions.

No discussion of the toxicity of cations would be complete without including something about sorption. Are cations adsorbed at the periphery of the cell or are they absorbed freely into the interior? Bodnar and Terenyi (1930) found that the uptake of copper by *Tilletia tritici* followed the Langmuir adsorption isotherm. Sussman and Lowry (1955) have strong evidence that dormant ascospores of *Neurospora* also adsorb cations. McCallan (1948), working with copper sulfate on spores of *Alternaria oleraceae* and *Monilinia fructicola,* could not fit his data to Langmuir's isotherm. Miller *et al.* (1953b) do not believe that silver and cerium are merely adsorbed. As evidence, they cite the enormous quantities of cations taken up by their fungus spores. The number of cationic molecules taken up, if merely adsorbed, would form layers several molecules thick on the spore surface. In addition, a pretreatment with silver does not interfere with the uptake of cerium and vice versa. Interference would be expected if uptake of these cations were strictly by adsorption. The work of Owens and Miller (1957) also points to penetration of cations into spores with practically no concentration on the cell walls. Rothstein and Hayes (1956), however, find that yeast cells bind both univalent and bivalent cations at the cell periphery. The binding is rapid and reversible, with the bivalent ions being held more firmly than the univalent ions. They tentatively identify the binding sites as phosphoryl and carboxyl groups. These sites are apparently separated from the interior of the cell by a permeability barrier. Rothstein and Hayes suggest that certain cations will have special affinities for specific binding groups on the cell surface: UO_2^{++} for carboxyl groups, Hg^{++} for sulfhydryl groups, and Zn^{++} for imidazole groups.

The evidence, then, is contradictory, but can be resolved. In organisms where there is a permeability barrier, adsorption is readily demonstrated. Where no permeability barrier exists or is destroyed by the toxicant, adsorption may play a role but is obscured by the free passage of cations into and out of the cell.

Another factor which confounds the sorption picture is the effect of metabolites leaking from the cell (McCallan and Wilcoxon, 1936). Miller and McCallan (1957) have shown that treating spores of *M. fructicola* with toxic doses of Ag^+ for as little as 1.2 minutes increases their outward loss of phosphorous compounds. The loss of phosphorous compounds from these silver treated cells is 20 times that of the untreated spores. This mass of organic material capable of complexing will undoubtedly have a profound influence on the final location of cations on or in the spore.

B. *Nonmetals*

1. *Sulfur*

Of the nonmetals, elemental sulfur is the most important fungitoxicant. In spite of its use as a pesticide for centuries, its method of toxic action is still a question for debate. Pollacci (1875) suggested that H_2S produced from sulfur is actually responsible for the fungitoxic action of sulfur. Young (1922) proposed pentathionic acid as the toxic product from sulfur. Pentathionic acid results from the interaction of H_2S and SO_2. The importance of pentathionic acid, SO_2, and SO_3 was largely discounted by Roach and Glynne (1928) and Wilcoxon and McCallan (1930). These workers found that the toxicity of these compounds is no more than could be accounted for by their hydrogen ion concentrations.

McCallan and Wilcoxon (1931) found that sulfur-sensitive spores produced large amounts of H_2S from sulfur. They reverted to Pollacci's idea that H_2S, which they found to be enzymatically produced, was the cause of sulfur toxicity. When they again tested the H_2S theory, Miller *et al.* (1953a) found that colloidal sulfur is as much as 50 times as toxic as H_2S to sulfur-sensitive fungi. H_2S, they now believe, cannot be the toxic agent responsible for the toxicity of sulfur. They concluded that sulfur poisons because it is an active hydrogen acceptor and so interferes in normal hydrogenation reactions of the cell. The intensity of H_2S production is a measure of the intensity of the hydrogenation process within the cell.

The theory of sulfur poisoning by competing for hydrogen seems plausible, but is still open to question. For example, methylene blue and ascorbic acid, both excellent hydrogen acceptors, are only weakly fungitoxic. Miller *et al.* (1953a) use as supporting evidence the work of Sciarini and Nord (1943) who concluded that, in *Fusaria*, elemental sulfur competes for hydrogen freed by dehydrogenases. Although Sciarini and Nord present such a conclusion, it is not supported by their data.

All of these ideas are possible. What Miller *et al.* (1953a) have actually shown is that H_2S produced within the spore is more toxic than H_2S in the ambient fluid. This is not sufficient evidence to discard H_2S as the source of sulfur toxicity. H_2S which ionizes primarily as HS^- may well poison metal-activated compounds just as metals poison sulfhydryl-activated compounds.

Miller *et al.* (1953a) also discount H_2S as a factor because they find the H_2S given off pretty well accounts for all the sulfur added to the system. However, the amount of H_2S bound *in situ* by the micro-quantities of metals in the spore would be so small that it would be practically undetectable.

McCallan and Miller (1956) made the significant discovery that fungi in reducing sulfur release one mole of extra CO_2 for each mole of H_2S produced. This contradicts Horsfall's (1956) suggestion that sulfur replaces oxygen in Krebs cycle intermediates. If this were so, then there should be less CO_2 than normal, rather than more. The production of CO_2 also indicates that elemental sulfur is not just competing for hydrogen, but appears to be acting as a strong oxidizing agent in the process of being reduced.

2. *Halogens*

Halogenated inorganic compounds are another group of useful toxicants. The commonest inorganic halogen compounds used as germicides are calcium or sodium hypochlorite. The toxicity of these compounds is directly correlated to the oxidizing power of their solutions. For example, Rideal and Evans (1921) showed that alkalinity which reduces the germicidal effect of hypochlorites also reduces their oxidizing power. The strength of the hypochlorites is expressed as "available chlorine," a measure of oxidizing power.

CaOCl reacts with water and CO_2 from the air as in Equation 5.

$$\begin{aligned} \mathrm{CaOCl} + \mathrm{HCO_3^-} &\rightarrow \mathrm{Ca\ HCO_3} + \mathrm{ClO^-} \\ \mathrm{ClO^-} &\rightarrow \mathrm{Cl^-} + \mathrm{O} \uparrow \end{aligned} \qquad \text{(Eq. 5.)}$$

Chlorine evolved from acidification of CaOCl, will also oxidize as in Equation 6.

$$\begin{aligned} \mathrm{Cl_2} + \mathrm{H_2O} &\rightarrow \mathrm{HCl} + \mathrm{HOCl} \\ \mathrm{HOCl} &\rightarrow \mathrm{HCl} + \mathrm{O} \uparrow \end{aligned} \qquad \text{(Eq. 6.)}$$

This is the method by which chlorine gas purifies water. Halogens may also act by halogenating metabolites, thus producing antimetabolites *in situ.*

3. *Formaldehyde*

Formaldehyde (HCHO) is widely used as a soil fumigant. Its action is primarily as a protein denaturant. The combination of formaldehyde with amino acids, as in the classic Sörenson formol titration (Equation 7), is representative of the way formaldehyde may combine with and denature proteins.

$$\underset{\text{(Zwitterion)}}{^+\mathrm{NH_3CH\ (R)\ COO^-}} + \mathrm{HCHO} \rightarrow \mathrm{CH_2O.\ NH_2\ CH\ (R)\ COO^-} + \mathrm{H^+} \qquad \text{(Eq. 7.)}$$

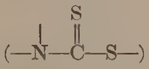
4. *Ammonia gas*

Ammonia fumigation has recently been used successfully in citrus storages for reducing fruit rots (Roistacher *et al.*, 1957). Ammonia, highly soluble in water with which it forms NH_4OH, probably poisons fungi by drastic changes in their internal pH.

IV. ORGANIC FUNGITOXICANTS

A. *Dithiocarbamates*

The greatest number of useful organic fungitoxicants has come from the dithiocarbamates. These compounds all have in common the grouping

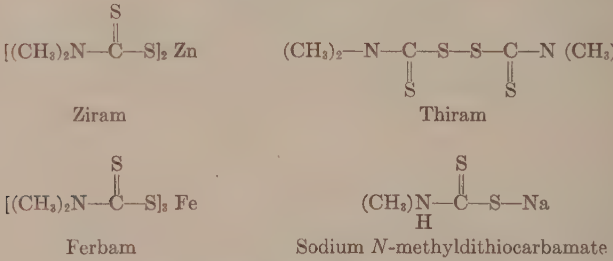


$$(\text{—}\overset{|}{\mathrm{N}}\text{—}\overset{\overset{\mathrm{S}}{\|}}{\mathrm{C}}\text{—S—})$$

One series, based on the patent issued to Tisdale and Williams (1934), is made from the alkaline interaction of CS_2 and simple amines. These compounds are the dialkyldithiocarbamates and thiuram sulfides. The second series, based on Hester's patent (1943), is made from the alkaline interaction of CS_2 and ethylenediamine. These are the ethylene bisdithiocarbamates.

1. *Dialkyldithiocarbamates and Thiuram Sulfides*

The most widely used of these materials are zinc dimethyldithiocarbamate (ziram), ferric dimethyldithiocarbamate (ferbam), and tetramethylthiuram disulfide (thiram). Recently, another member of the family, sodium *N*-methyldithiocarbamate, has been finding use as a soil fungicide. The structures of these compounds are given in Fig. 7.



$[(CH_3)_2N\text{—}\overset{\overset{S}{\|}}{C}\text{—}S]_2\ Zn$

Ziram

$(CH_3)_2\text{—}N\text{—}\underset{\underset{S}{\|}}{C}\text{—}S\text{—}S\text{—}\underset{\underset{S}{\|}}{C}\text{—}N\ (CH_3)_2$

Thiram

$[(CH_3)_2N\text{—}\overset{\overset{S}{\|}}{C}\text{—}S]_3\ Fe$

Ferbam

$(CH_3)\underset{H}{N}\text{—}\overset{\overset{S}{\|}}{C}\text{—}S\text{—}Na$

Sodium *N*-methyldithiocarbamate

FIG. 7. Structures of ziram, thiram, ferbam, and sodium-*N*-methyldithiocarbamate.

The history of the development of these compounds and their performance in the laboratory has been reviewed thoroughly by Klöpping (1951), Horsfall (1956), and McCallan (1957).

The two most important clues to the toxic action of the dialkyl dithiocarbamates and the thiuram disulfides are (a) their formation of metal chelates and (b) their polymodal dosage response. The first property is a well-known one, commonly used in analytical chemistry. The second was first reported by Dimond *et al.* (1941), for thiram. This latter effect, also called "inversion" and "quenching," is described in detail and logically explained by Goksøyr (1955).

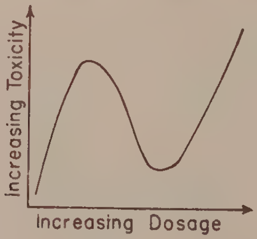
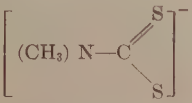


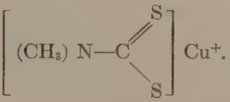
FIG. 8. Polymodal dosage response curve, or inversion of toxicity.

Inversion of toxicity is pictured in Fig. 8.

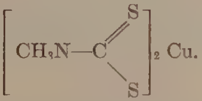
The zone of inversion, where increasing dosage actually depresses toxicity, has been particularly hard to explain. Goksøyr's explanation is as follows. The dimethyldithiocarbamate ion,



combines with minute traces of copper in the media to give the highly toxic 1 : 1, or half chelate,



This is the toxic species. As the amount of copper in the media is constant, the addition of more dithiocarbamate forms the less toxic 1 : 2, or full chelate,



Toxicity falls. As more dithiocarbamate is added, the intrinsic toxicity of the dimethyldithiocarbamyl ion exerts itself, acting perhaps by binding essential metallic micronutrients. Thus toxicity increases again.

Goksøyr demonstrated that yeast cells reduce thiram to dimethyldithiocarbamyl ions. Hence, thiram activity and its polymodal dosage response curve is identical to those of the dialkyldithiocarbamates.

As will be seen later, *N*-methyldithiocarbamate has an action more like the ethylenebisdithiocarbamates than like the dialkyldithiocarbamates.

2. *Bisdithiocarbamates*

This group of compounds arose around disodium ethylenebis (dithiocarbamate), now called nabam (Fig. 9).

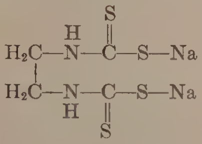


FIG. 9. Nabam.

The history of their development and the laboratory results of structure-activity studies are given in Barratt and Horsfall (1947), and Klöpping (1951).

What is the basis for their activity? Barratt and Horsfall (1947) proposed that since these compounds readily release H_2S, they poison by means of the H_2S evolved. Rich and Horsfall (1950) demonstrated that H_2S could not account for the toxicity of nabam. The clue to the currently accepted mechanism was noted in the title of the paper by Dimond *et al.* (1943), in the first report on nabam. They called it a "water-soluble fungicide with tenacity." This anomaly puzzled them. In the data of Barratt and Horsfall appeared another clue. Zineb, the zinc salt of nabam, becomes more toxic upon aging. Klöpping (1951) blazed the trail which led to the present theory. Realizing that diisothiocyanates could form from bisdithiocarbamates, he tested the fungitoxicity of the diisothiocyanates. Klöpping's biological tests showed that the diisothiocyanates have the same biological spectrum as the bisdithiocarbamates. By "biological spectrum" he meant that different fungi have the same relative susceptibility to both types of compounds. In addition, the diisothiocyanates are much more toxic than the corresponding bisdithiocarbamates. Data from Klöpping (Table I) are illustrative.

TABLE I

FUNGITOXICITY DATA FROM KLÖPPING ILLUSTRATING SIMILARITY BETWEEN THE "BIOLOGICAL SPECTRA" OF NABAM AND ETHYLENE DIISOTHIOCYANATE

	Minimum p.p.m. completely inhibiting growth			
Compound	*B. cinerea*	*P. italicum*	*A. niger*	*R. nigricans*
Nabam	0.1	0.1	0.5	20.0
Ethylenediisothiocyanate	0.05	0.02	0.05	10.0

Klöpping concluded that the ethylenebisdithiocarbamates act by releasing diisothiocyanates, which do the poisoning. The diisothiocyanates being extremely reactive and unstable, are hard to detect as breakdown products.

Shortly after, Ludwig and Thorn (1953) looking for insoluble (tenacious) compounds from the oxidation of nabam, found that ethylenethiuram monosulfide is a major product of nabam oxidation. They found that ethylenethiuram monosulfide (Fig. 10) is highly fungitoxic.

Ethylenethiuram monosulfide they concluded to be the toxic breakdown product accounting for both the tenacity and toxicity of nabam. In a later paper, Ludwig *et al.* (1955) reported that metallic ions catalyze the formation of isothiocyanates from ethylenethiuram monosulfide, They could demonstrate this only in a nonaqueous medium (chloroform).

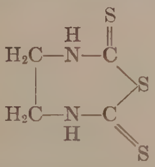
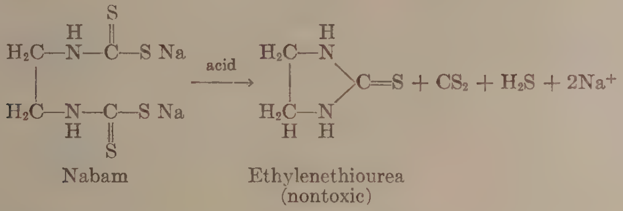

H
H_2C—N—C
S
S
S
H_2C—N—C
H

FIG. 10. Ethylenethiuram monosulfide.

In return, Sijpesteijn and van der Kerk (1954) confirmed the production of ethylenethiuram monosulfide from nabam.

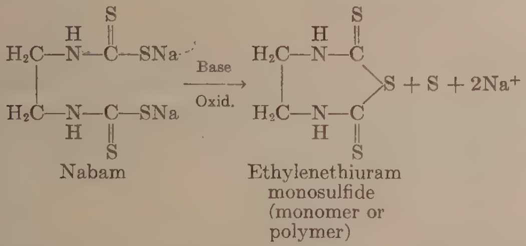
The present theory of nabam action may be summarized from the literature. Freshly prepared, unoxidized nabam is presumed to be essentially nontoxic.

The acidic breakdown of nabam (Lopatecki and Newton, 1952) proceeds as in Equation 8.


acid
C=S + CS_2 + H_2S + $2Na^+$
Nabam
Ethylenethiourea
(nontoxic)

(Eq. 8.)

Basic hydrolysis and oxidation of nabam proceeds in another fashion (Equation 9).

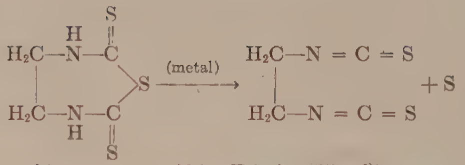

Base
Oxid.
S + S + $2Na^+$
Nabam
Ethylenethiuram
monosulfide
(monomer or
polymer)

(Eq. 9.)

Ethylenethiuram monosulfide in turn breaks down to release ethylenediisothiocyanate (Equation 10).

The diisothiocyanates, highly reactive and unstable, apparently combine with sulfhydryl groups to act as nonspecific poisons.

One of the unresolved problems of nabam toxicity is the nature of the gaseous toxicant which it evolves. This unidentified gas was first



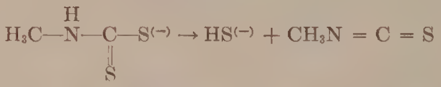
(Eq. 10.)

Ethylenethiuram monosulfide Ethylenediisothiocyanate

detected by Rich and Horsfall (1950), who demonstrated that the gas could not be H_2S or SO_2. They did not consider CS_2 as a possibility because it is a relatively weak toxicant. In 1951, Cox *et al.* showed that CS_2 and ethylenediamine evolve from nabam hydrolysis. They proposed that these two gases are the toxic vapors, recombining within the spore to give nabam. Lopatecki and Newton (1952) argued that the toxic vapors are simply CS_2 evolved from nabam. Later work on the problem by Weed *et al.* (1953) practically ruled out CS_2 or ethylenediamine as factors. These workers also doubted the possibility of the toxic gases being a diisothiocyanate. Weed *et al.* (1953) conclude that "an unidentified volatile constituent is involved" in the toxicity of nabam. There the problem stands.

Two other types of compounds must be included here although they are not ethylenebisdithiocarbamates. These are sodium *N*- methyldithiocarbamates and the rhodanines.

Sodium *N*-methyldithiocarbamate, in spite of structural similarity to the dialkyldithiocarbamates, is also a source of methylisothiocyanate (Klöpping, 1951), as in Equation 11. Sodium *N*-methyldithiocarbamate

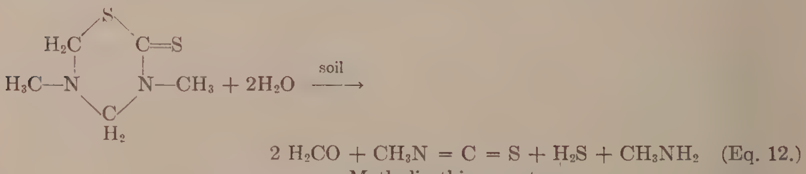


$$H_3C{-}\overset{H}{N}{-}\underset{\underset{S}{\|}}{C}{-}S^{(-)} \rightarrow HS^{(-)} + CH_3N = C = S \qquad \text{(Eq. 11.)}$$

N-Methyldithiocarbamate ion Methylisothiocyanate

is used as a soil fungicide under the trade names of "Vapam" or "VPM."

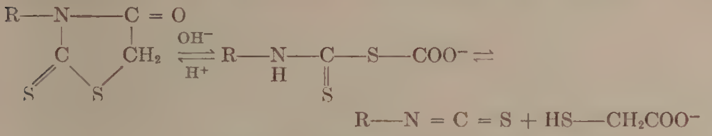
3,5-Dimethyltetrahydro-1,3-5,2H-thiadiazine-2-thione, the active ingredient of the soil fumigant "Mylone," also breaks down to give methylisothiocyanate as in Equation 12 (Torgeson *et al.* 1957).



$$2\ H_2CO + CH_3N = C = S + H_2S + CH_3NH_2 \qquad \text{(Eq. 12.)}$$

Methylisothiocyanate

Another group of related fungitoxicants, the rhodanines, was described by van der Kerk *et al.* (1953). The structure-activity of the rhodanines was also reported in 1953 by Brown and her co-workers (1953a, b). Van der Kerk reports (1956) that the rhodanines also are isothiocyanate fungicides and act according to Equation 13.


(Eq. 13.)

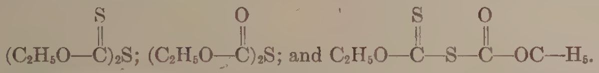
B. *Other Organic Sulfur Compounds*

Although the dithiocarbamates have been the most useful and interesting of the organic sulfur fungicides, others also have been found to be fungitoxic.

Some of these are the ethylenethioureas, the xanthates, 2-mercaptobenzothiazole, the thiobisphenols, and those toxicants having a trichloromethylthio group.

Rich and Horsfall (1954b) noted that the *N*-alkylethylenethioureas increased in fungitoxicity with increasing length of alkyl chain. Fungitoxicity is maximal at the 8 carbon homologue and decreased as the chain lengthens beyond 8 carbons. Ross and Ludwig (1957) demonstrated that this series probably acts as physical toxicants exhibiting the Ferguson effect.

The xanthates, long used as disinfectants, were thoroughly investigated by Davies and Sexton (1946). They concluded that sulfur atoms on the $C = S$ groups were not essential to the toxicity of xanthates. This conclusion was based on the essentially equal fungitoxicity of the following three compounds:


$$(C_2H_5O{-}\overset{\overset{S}{\|}}{C})_2S;\ (C_2H_5O{-}\overset{\overset{O}{\|}}{C})_2S;\ \text{and}\ C_2H_5O{-}\overset{\overset{S}{\|}}{C}{-}S{-}\overset{\overset{O}{\|}}{C}{-}OC{-}H_5.$$

2-Mercaptobenzothiazole, although it is somewhat fungicidal (Marsh, 1938), has found its principal use as a synergist with dialkyldithiocarbamates in proprietary mixtures sold as "Vancides."

The thiobisphenols will be discussed under phenolic fungicides. The trichloromethylthio compounds will be discussed under heterocyclic nitrogen compounds because of the interest in captan, the tetrahydrophthalimide derivative.

C. *Heterocyclic Nitrogen Compounds*

Horsfall and Rich (1951) reported on the structure-activity relationships in a large group of heterocyclic nitrogen compounds. Many of these compounds are extremely fungitoxic. Their toxic mechanisms, however, differ widely. Certain of them, such as the ethylenethioureas just discussed, the nitrosopyrazoles (Rich and Horsfall, 1952), and the tetrahydropyrimidines (Rader *et al.*, 1952), probably act as physical toxicants. Others, such as the 2-imidazolines have been reported to be competitive inhibitors of enzymes (West and Wolf, 1955). The action of acridine was discussed in an earlier section. Two other mechanisms may also be important to the fungitoxicity of heterocyclic nitrogen compounds. These latter two mechanisms may best be presented by discussing two compounds for which these mechanisms have been proposed. The compounds are 8-quinolinol (oxine), and *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide (captan). Oxine has been introduced in the previous section on chelation, and will be discussed first.

Oxine is the cornerstone for the theories of toxic mechanisms involving chelation. As mentioned earlier, the idea that oxine takes needed metals from organisms was called into doubt by evidence that the metal oxinates, particularly cupric oxinate, is more toxic than oxine itself (Mason, 1948). This evidence was further supported by Anderson and Swaby (1951), who found that oxine is a copper-deficient medium is only $^{1}/_{48}$ as toxic as it is in a copper-sufficient medium. Copper must be present, therefore, for maximum fungitoxicity of oxine. Not only must there be copper present but the oxine must be able to form a chelate with it. For example, at a pH below which oxine chelates, it is no longer toxic, regardless of the presence of copper. The formation of a copper oxinate, then, appears to be essential to the fungitoxicity of oxine.

Complications arose. Block (1957) showed that the fungitoxicity of copper oxinate can be reversed not only with excess copper but also with excess oxine. Albert *et al.* (1953) had reported earlier that the antibacterial action of oxine can be quenched by an excess of oxine—the polymodal dosage response again. They proposed that excess oxine inhibited the formation within the cell of the 1 : 1, or half chelate which they suggested as the toxic form of oxine. Reversal by excess metal, they argued, was caused by inhibiting the 1 : 2 or full chelate in the ambient fluid. They pictured the process as follows. Oxine, because of water solubility, penetrates into cells best as the fully chelated metal complex which is less polar. Once inside the cell, the half chelated complex is required for toxicity. This half chelate may arise from the full chelate either by dissociation, or by the action of a hypothetical enzyme. Their theory is illustrated by Equation 14. Excess metal in the ambient fluid would drive the last equation to the left, reducing the amount of fully chelated oxine needed for penetration. Excess oxine within the cell would drive the last equation to the right, reducing the concentration of the toxic species, the half chelate. Other chelators in

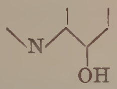
$$\text{Oxine}^- + \text{Metal}^{++} \rightleftharpoons \underset{\substack{\text{Half chelate}\\ \text{(Toxic within cell)}}}{\text{(Oxine) Metal}^+} \qquad \text{(Eq. 14.)}$$

$$\text{(Oxine) Metal}^+ + \text{Oxine} \rightleftharpoons \underset{\substack{\text{Full chelate}\\ \text{(Needed for permeation)}}}{\text{(Oxine)}_2\ \text{Metal}}$$

excess will also cause reversal of oxine, ostensibly by competing for metals (Zentmyer and Rich, 1956; Sijpesteijn *et al.*, 1957). Block (1955) demonstrated that in addition to the ability to chelate, analogues of oxine must also be lipoid-soluble to be fungitoxic.

There are at least two discrepancies in the theory that should be noted. The first is that unchelated oxine enters cells quite readily (Greathouse *et al.*, 1954). In fact, the theory for the reversal of toxicity by excess oxine depends on its entry into the cell. The second discrepancy is that washing with water will revive spores poisoned with oxine in a metal-deficient medium, but will not revive copper oxinate-treated spores (Block, 1956). Washing with 0.1 *N* HCl is needed to revive the latter. These anomalies imply that unchelated oxine, which is toxic, acts in an area of the cell which does not require high lipoid-solubility to be entered. This action of oxine may well be metal-robbing. The chelated oxine acts at a site which requires high lipoid-solubility. This deeper site may well be within a microsome or the nucleus. The action of the half chelate may be the blocking of functional sites as suggested earlier.

Other workers have different views about the toxicity of oxine. Sexton (1953) considers it likely that oxine is fungitoxic because of its phenolic properties. Gale and Folkes (1957) find that oxine is one of the best inhibitors of the incorporation of glycine in disrupted staphylococcal cells. They consider that the



portion of oxine structurally resembles, and competes with natural components of the system they were studying. They base this conclusion on two observations: (a) their system is stimulated by 6-amino-4-hydroxy derivatives of benzimidazole or benzotriazole; and (b) their system is not inhibited by the chelator ethylenediamine tetraacetic acid.

The next heterocyclic nitrogen compound to be discussed is captan (Fig. 11). The discussion of captan toxicity has centered on the importance of the —SC(Cl)$_3$ group. Included here, then, will be some analogues of captan that are not heterocyclic nitrogen compounds, but do have the —SC(Cl)$_3$ group.

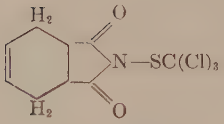


FIG. 11. Captan.

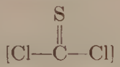
The fungitoxicity of captan was first reported by Kittleson (1952). He considered the fungitoxic group to be

$$>N{-}S{-}(C(Cl)_3).$$

Horsfall and Rich (1953) presented evidence that —CO—NR—CO— could be the toxic grouping of captan. They based their conclusion on the similarity of the dosage-response slopes of captan and the highly fungitoxic antibiotic cycloheximide. Cycloheximide has the —CO—NH—CO— group but not —$SC(Cl)_3$. Horsfall and Rich assigned to the trichlororomethylthio group the function of getting the tetrahydrophthalimide portion of captan into the cell. *N*-nitrosophthalimidine (Ladd, 1945) is another highly fungitoxic compound with the —CO—NR—CO— group but no —$SC(Cl)_3$. The fungitoxicity of this compound was also used by Horsfall and Rich to support their theory. Horsfall and Rich (1954) pointed out that electronegative groups adjacent to a sulfur atom enhance activity. The electronegative carbonyl groups in captan would draw electrons from the nitrogen atom, making the N–S bond less stable.

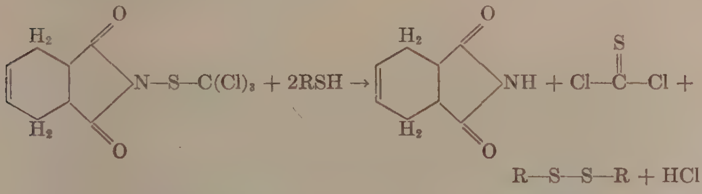
Uhlenbroek and Koopmans (1957) found that —$SC(Cl)_3$ alone did not make a compound fungitoxic. They reported that $H_3CSSC(Cl)_3$ is nonfungitoxic, $C_6H_5SCC(Cl)_3$ is slightly fungitoxic, while 2-$NO_2C_6H_4$-$SSC(Cl)_3$ is very fungitoxic. In addition, they found that —$C(Cl)_3$ could be replaced without destroying fungitoxicity completely. For example, the ED50 of $H_3CC_6H_4SO_2SC(Cl)_3$ to *Fusarium culmorum* is in the range of 0.315 to 1.0 mg./liter, very fungitoxic. Replacing the —$C(Cl)_3$ with a —CH_3 results in a compound with an ED50 in the range 1.0–3.1 mg./liter, still quite fungitoxic. Uhlenbroek and Koopmans found that the more electronegative groups on R in $RSSC(Cl)_3$, the more fungitoxic the analogue. In $RSO_2SC(Cl)_3$, however, the analogues are quite fungitoxic, regardless of the electronegativity of R. They concluded that the hypothesis of increased fungitoxicity by electronegative groups adjacent to an S atom may apply for $RSSC(Cl)_3$ but not for RSO_2-$SC(Cl)_3$. According to Ingold (1953), however, —SO_2R is a very strong electronegative group in itself. Changes in R, therefore, may not give good evidence for or against the electronegativity theory, when —SO_2R is adjacent to a sulfur atom.

Another theory of captan action was presented by Lukens and Sisler (1958). They found that captan interacts with sulfhydryl compounds, such as cysteine, to give thiophosgene



$$[\text{Cl}-\overset{\overset{\text{S}}{\|}}{\text{C}}-\text{Cl}]$$

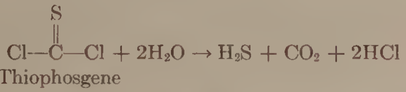
as in Equation 15.



(Eq. 15.)

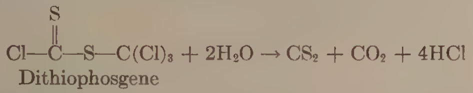
A clue to this breakdown is in the odor of formulated captan which is that of thiophosgene.

The thiophosgene formed may react with water (Equation 16).



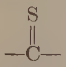
$$\underset{\text{Thiophosgene}}{\text{Cl}-\overset{\overset{\text{S}}{\|}}{\text{C}}-\text{Cl}} + 2H_2O \rightarrow H_2S + CO_2 + 2HCl \qquad \text{(Eq. 16.)}$$

Thiophosgene also polymerizes to give dithiophosgene which in turn may react with water (Equation 17).



$$\underset{\text{Dithiophosgene}}{\text{Cl}-\overset{\overset{\text{S}}{\|}}{\text{C}}-\text{S}-\text{C(Cl)}_3} + 2H_2O \rightarrow CS_2 + CO_2 + 4HCl \qquad \text{(Eq. 17.)}$$

Lukens and Sisler proposed that the toxicity of captan is produced by the highly reactive thiophosgene resulting from the breakdown of captan by sulfhydryl groups. They were also able to demonstrate this breakdown by reacting captan with yeast cells. They concluded that essentially all of the toxicity of captan to yeast can be accounted for by the $—S—C(Cl)_3$ group. Thiophosgene produced *in situ* would ostensibly poison by combining with free sulfhydryl, amino, hydroxyl, and perhaps carboxyl groups. In addition, thiophosgene could form



$$-\overset{\overset{\text{S}}{\|}}{\text{C}}-$$

bridges between properly spaced groups within the cell.

The production of thiophosgene is undoubtedly of importance to captan activity. Whether or not the captan acts simply as a thiophosgene producer remains doubtful. If, as Uhlenbroek and Koopmans (1957) reported, some —S—C(Cl)$_3$ compounds are nontoxic, and if replacing —C(Cl)$_3$ does not destroy fungitoxicity, then thiophosgene production may not be the whole story of —S—C(Cl)$_3$ compounds. Captan, which loses its chlorine atoms readily, may well form the *N*-chlorotetrahydrophthalimide as a transitory intermediate, analogous to diisothiocyanates formed in nabam breakdown. That the *N*-chloro derivative may form is supported by Orwoll (1954). He presented the following possibility (Equation 18) for the breakdown of a captan-type molecule.

$$Ar - SCCl_3 \rightarrow CS\,Cl_2 + Ar\,Cl \qquad \text{(Eq. 18.)}$$

Although *N*-chlorotetrahydrophthalimide is too unstable to test, its analogue, *N*-chlorosuccinimide, is a well known and highly active germicide. The phthalimide itself could be very fungitoxic when released *in situ*. The breaking of the N—S bond would leave the N position free to combine with any reactive grouping within the cell. Captan, then may poison by releasing highly reactive tetrahydrophthalimide radicals, thiophosgene, and chlorine.

So much for the heterocyclic nitrogen compounds, a particularly rich source of fungitoxicants.

D. *Quinones*

Quinones are among the most powerful of the agricultural fungicides. The two most commonly used in practice are both chlorinated compounds. These are 1,4-tetrachlorbenzoquinone (chloranil), and 2,3-dichloro-1,4-napthoquinone (dichlone).

Chloranil has found its use primarily as a seed protectant. As a foliage fungicide it usually fails to protect, being readily photolysed to nontoxic products.

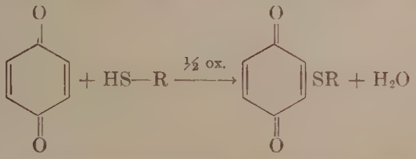
Dichlone, much less readily photolysed, is widely used as a foliage spray. It has been particularly successful against apple scab. The potency of the quinones may be illustrated by the usage of dichlone for controlling apple scab. Most protective fungicides for apple scab control require 1 to 3 lb. of formulation per 100 gallons. Dichlone is used successfully at a dosage of ⅛ to ¼ lb. per 100 gallons.

Owens (1953a) points out a number of ways by which quinones may poison spores. As α,β-unsaturated compounds they may combine with sulfhydryl groups (Equation 19).
They may also interact with amino groups.

Quinones may also poison by rerouting electron transfer systems as described in an earlier section.

McNew and Burchfield (1951) found the following order of increasing fungitoxicity: anthraquinone < benzoquinone < phenanthraquinone

< naphthoquinone. Halogenation, while decreasing water solubility and phytotoxicity, increases fungitoxicity. Chlorination improves fungitoxicity the most, bromination next, and iodination least. Alkylation ortho to carbonyl groups reduces fungitoxicity, presumably by steric hindrance.



(Eq. 19.)

A point of interest is that the quinonoid structure may not be necessary to the activity of these compounds. For example, Owens (1953b) reports that 2,5-dichlorohydroquinone is almost as toxic as chloranil. Of course, data of Rich and Horsfall (1954a) suggest that the hydroquinone may be oxidized to the quinone by fungi.

E. *Phenolic Compounds*

Phenolic compounds have been of interest as germicides since the introduction of carbolic acid into surgery by Lister in the last century. Phenols as fungitoxicants or germicides have been particularly useful in medicine and industry rather than in agriculture. One of the most widely used germicides in medicinal preparations is hexylresorcinol. Various derivatives of cresol and phenylphenol are used as wood preservatives. Sodium dinitro-*o*-cresylate (Keitt, 1939) has been used as a spray on orchard floors to kill overwintering forms of fungi which cause fruit diseases.

One of the significant contributions to the structure-fungitoxicity relationships of phenols was made by Shirk (1954). He found that alkylating both positions ortho to the phenolic —OH greatly reduced fungitoxicity. In addition, *o*-chloro-6-alkylphenols are less fungitoxic than the corresponding *o*-alkylphenols (Shirk and Corey, 1952). Shirk explains this effect as steric hindrance. He bases his suggestion on the report of Stillson *et al.* (1945) who found that phenols substituted 2,6 with large alkyl groups gave reactions not at all like typical phenols. These they called hindered phenols. Interfering with the reactivity of the phenolic —OH, then, profoundly affects fungitoxicity.

Another group of phenolic fungicides of interest are the bisphenols. The germicidal action of these compounds was first reported by Bechold and Ehrlich (1906). Marsh *et al.* (1945), and Marsh and Butler (1946) found bisphenols to be highly effective mildew preventives on cloth. Their best material was 2,2′-methylenebis (4-chlorophenol) also known as G-4, preventol GD and dichlorophene (Fig. 12).

Goldsworthy and Gertler (1949) reported this compound to be an excellent foliage fungicide for peach trees.

The corresponding thiobisphenols are also effective fungicides. Here, the methane bridge is replaced by a sulfur atom. Pfleger *et al.* (1949), found that the thiobisphenols are highly toxic *in vitro* to fungi causing human diseases. Schraufstätter *et al.* (1949) reported 2,2′-thiobis (4-chlorophenol) as a particularly good antimycotic. Horsfall and Rich (1950) showed Schraufstätter's compound to be an excellent foliage protectant against apple scab. Unfortunately, it damaged the fruit.

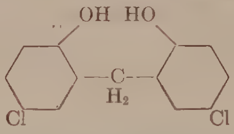


FIG. 12. 2,2′-Methylenebis(4-chlorophenol).

Cade and Gump (1954) summarized the results of structure-activity research with bisphenols. Activity is increased by halogenation of each ring in the 4-positons. Any more halogenation decreases activity. The phenolic —OH's are most effective ortho to the bridge linking the rings. The type of bridge between the two rings is not too important. The bridge must be small enough, however, to prevent spreading the hydroxyls too far apart. This latter evidence indicates that chelation may play a part in the toxicity of the bisphenols.

There are many other kinds of fungicides, such as salicylates and benzoates, that have been considered phenolic toxicants. The large group of phenolic toxicants are usually considered as nonspecific, protoplasmic poisons.

F. *Halogenated and Nitrated Aromatic Compounds*

The halogenated aromatic compounds are usually toxic because they readily release nascent halogens. This is undoubtedly the mechanism of action of such compounds as chloramine-T and *N*-chlorosuccinimide. The freed chlorine may act to form oxidizing $HClO^-$ as in the case of the hypochlorites. Another kind of action may be a direct halogenation of a metabolite to form an antimetabolite.

Nitrated aromatic compounds most potent as fungicides are usually either halogenated or they are phenols. One of these compounds, 2,4-dinitro-*o*-cresol was mentioned in the previous section. Another is 2,4-dinitrophenol, a powerful uncoupler of phosphorylation. Of the halogenated nitro-aromatics, one of the most powerful fungicides is 1-fluoro-3-bromo-4,6-dinitrobenzene (Finger *et al.*, 1955). The chlorinated nitrobenzenes have been of continuing interest since they were first described as fungitoxicants (Brown, 1935). Pentachloronitrobenzene and tetrachloronitrobenzene have found use both as soil fungicides and for the control of *Botrytis* sp. on plant parts. These compounds prevent sporulation, and are vapor-phase fungistats which do not prevent spores from germinating but do stop hyphal growth (Reavill, 1954). Horsfall (1956) suggests that compounds of this type are antimitotics. It may be that pentachloronitrobenzene and tetrachloronitrobenzene act specifically as competitive inhibitors for inositol, a required growth substance for many fungi.

G. *Other Organic Fungitoxicants*

The list of fungitoxic organic compounds is a long one. They cannot all be mentioned in one short chapter. Such fungitoxicants as quaternary ammonium compounds were mentioned in the section as surfactants. These have been tried for the control of plant diseases (Howard and Keil, 1943).

Not yet mentioned are the aliphatic fungitoxicants. These are of not too much importance and most probably act as nonspecific, physical toxicants. The organometallic fungicides have been of particular interest, so should not be omitted. Suffice it to say that many organometallic compounds, such as the organic mercurials, are more powerful than the inorganic compounds of the same metals. Of interest in this regard is tin, not very fungitoxic in inorganic combinations, but producing some highly fungitoxic organic compounds (van der Kerk and Luijten, 1954).

V. FASHIONING FUNGICIDES

A. *Success of Screening Programs*

The artistry of the synthesizing chemists combined with the carefully designed screening programs of the biologists has given us a wealth of new and highly effective fungicides in recent years. The methods used for screening are as various as the laboratories so engaged. Although the selection of compounds to be screened is usually empirical, the tests to which the compounds are subjected are based on knowledge of plant diseases. Regardless of the method, there is very little question that most of the commercial fungicides now in use would have been selected by any of the presently operating screening programs. The screens work.

Concomitant with the selection of candidate fungicides, the screening programs have resulted in masses of structure-activity data. These data have been carefully scrutinized for "philosopher's stones" which will predict fungitoxic configurations. The literature abounds with such studies. These have been of use in predicting the activity of closely related compounds, and in a few cases have predicted the activity of untested configurations.

B. *Present Holes in Screening Programs*

One of the main problems plaguing searchers for fungicides is the field failure of compounds which look promising in the laboratory. The present laboratory programs will pick all the winners, but, in the process, they also pick too many losers. Field testing is expensive.

Why do compounds look good in the laboratory and yet often are of no value for protecting plants in the field? Some of these factors involve the physical form of the fungicide. One of these physical factors of primary importance is particle size. A great number of studies have demonstrated the relationship between particle size and both fungitoxicity and tenacity. In order for fungitoxic compounds to succeed in the field, they must not only be in a proper physical form but also they must be carefully formulated. Burchfield and Goenaga (1957) have recently shown that even such an old and widely used fungicide as Bordeaux can be made more effective by formulation with certain wetting agents. Copper oxide succeeded as a foliage fungicide only after each particle was coated with gum arabic. The gum arabic coating prevented the agglomeration and consequent poor performance of the copper oxide particles. This made the difference between success and failure.

Very often candidate fungicides fail because they have not been subjected in the laboratory to the extremes of weathering encountered in the field. Although laboratory testing ordinarily includes a washing or rain test, the compounds are not usually subjected to other weathering factors. Some of these additional factors are wind whipping of leaves, leaf expansion, high intensities of actinic light, long periods of continuous high humidity with attendant carbonation and oxidation, and periodic wetting and drying which would tend to increase hydrolysis. These factors, any of which may be critically important to field performance, are seldom or never tested in the laboratory. Fungitoxic compounds passing the rain test should be subjected to other weathering factors before being field tested.

Fungitoxicants may be lost not only by physical action but also through biological activity. The compounds may be degraded either by leaf emanations or by microbiological activity. Antibiotics are notoriously subject to degradation by soil microorganisms. It is interesting to note that biological degradation may actually make a compound more toxic.

Byrde and Woodcock (1953) report such a case. They found that 1:4-diacetoxy-2:3-dichloronaphthalene, weakly toxic, becomes highly toxic when mixed with an enzyme preparation of high acetyl-esterase activity. Byrde and Woodcock suggest that the esterase changes this compound into the corresponding hydroquinone. Horsfall (1956) suggested that a phenol oxidase converts the hydroquinone to the quinone.

The pitfalls of a candidate fungicide are many and varied. It should be possible, however, to devise laboratory tests to eliminate many of the compounds which now use up time, space, and money, failing in the field.

C. *Future of Fungicides*

What is the future of fungicides? The future of fungicides is excellent. We become discouraged and go through periods of "agonizing reappraisal" only because our ignorance is so profound. We cannot make sense of the rapidly accumulating data. This chapter and others like it were written with the hope of illuminating signposts. Only by extrapolation from existing knowledge can we expect to find a "field theory" of fungicide action, if such exists. Without question, the many pathways pointed out by structure-activity studies eventually will become usable roads.

Should we continue to search for better fungicides? We need only to look to the history of insecticides for an affirmative answer. The discovery of the insecticidal properties of DDT, the chlorinated hydrocarbons, and the phosphates, has given us insecticides many times more powerful than the old. So it will be with fungicides.

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CHAPTER 15

Nematocides

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The science of plant pathology was concerned primarily with the fungi during the first 50 years. Then the bacteria had a short period of prominence. This did not last long, however, because fungi were still more numerous as pathogens. Bacteria were pushed into the background, too, by the viruses which have been demanding increasingly more attention.

The nematodes are not "newcomers" as plant pathogens, the wheat nematode was known as a plant pathogen in 1744 and other important species were described in the 19th century. Present-day general acceptance of nematodes as important plant pathogens was arrived at slowly. Numerous reasons could be advanced to account for the seeming reluctance of plant scientists to embrace nematodes as plant pathogens. Perhaps the fact that they are animals and have been the concern of the zoologist, particularly the parasitologist, has tended to place the plant pathogenic nematodes in the unique position of being unclaimed, until recently, by any organized scientific discipline.

However, throughout this long period of general apathy toward the plant parasitic nematodes, their cause was proclaimed far and wide by a handful of devoted specialists in nematology who were concerned primarily with the importance of nematodes as destroyers of cultivated plants.

As the importance of many types of nematodes as plant pathogens was recognized, plant pathologists became more concerned with them. The result is that we have a new race of plant pathologists—those concerned with animals that are plant pathogenic. Moreover, we still have the zoologist specializing in nematology and contributing much of the fundamental information concerning systematics, morphology, physiology, and ecology of the soil inhabiting nematodes. The problem is of great magnitude and presently requires the additional knowledge that will result from the cooperation of these animal and plant scientists.

I. The Problem of Proving Pathogenicity

Nematologists have struggled again over the same old rough ground traversed by the mycologists, bacteriologists, and virologists. How can one prove that a nematode is a pathogen? Nematologists inherited agar techniques and petri plates from their predecessors but little else that was significant in their search for the elusive proof.

The extreme difficulty of obtaining pure cultures of nematodes has made the proof most difficult. Without proof, many plant pathologists have been loath to accept the pathogenicity of many species. They have accepted constant association as sufficient evidence for pathogenicity of such obvious nematodes as the root knot nematode and the golden nematode. ·

Nematocides have contributed enormously to the proof needed by the nematologists, who were not able, at least until recently, to culture the nematodes, add them to plants, produce the disease, and reisolate the original nematode. The use of nematocides to kill them has given a type of inverted proof. Constant association made this reasonably easy. Nematocides killed off the nematode and, thus, interrupted the constant association. This was nearly as good proof as Koch demanded. At least it was good enough to set many men baying down the trail of nematodes as plant pathogens. The subject grows apace.

II. What a Nematocide Is

The term nematocide is restricted in this discussion to chemicals with nematode-killing properties that have been used or are currently used to reduce populations of plant pathogenic nematodes.

In addition to nematocidal activity, useful nematocides must possess special properties with regard to phytotoxicity, diffusion in soil, water solubility, vapor pressure, and cost. The chemicals that are here considered as nematocides are not specific in their toxic action. They usually kill all of the nematodes that are present in the area of their activity. Some nematocides are fungicidal, insecticidal, and herbicidal as well, and could equally well be referred to as biocides since they partially or completely sterilize the soil.

In disease complexes involving nematodes and fungi or bacteria there may be secondary benefits from the application of nematocides. Generally, too, the benefits of treatments with nematocides are measureable in terms of increased crop yield, quality, and better plant growth. In practice, it is expected that the use of nematocides will increase the profit to the user. Some investigators suggest that a soil fumigation treatment with a nematocide should increase yield or quality of the product sufficiently to return to the grower an amount equal to twice the cost of the treatment. Taylor (1951) says that it is not economical to fumigate soil for nematode control unless the gross value of the crop is at least $375 per acre. These are matters that can only be decided by individual growers, since there are many economic factors that determine whether or not a nematocide is profitable. There are situations where a nematocide is not profitable even though it more than doubles the yield of the crop, as in California when sugar beet yields have been doubled but the yield on treated land is still not at an economic level. In other situations there may be no significant increase in total yield, but the quality is increased to profitable levels. This has been evident in such crops as carrots and potatoes (Taylor, 1951). One of the attractive features of nematode control by nematocides is that the benefits of the treatment are immediately obvious to growers in terms of net profit. This is no doubt responsible for the fairly rapid adoption of nematocides once they became available at relatively low cost.

III. Historical

The application of chemicals to the soil to control plant parasitic nematodes was undertaken by a number of investigators because crop failures were associated with the presence of these pests. Kühn (1881) attempted control of the sugar beet nematode, *Heterodera schachtii* Schmidt, 1871, with a number of chemicals and concluded that the most promising was carbon bisulfide. However, this chemical was not entirely satisfactory because of the high cost of the treatment and the relatively unsatisfactory results. It is likely that Kuhn used carbon bisulfide because of its then widespread use against grape *Phylloxera.* Neal (1889) applied carbon bisulfide to the soil as well as numerous combinations of tobacco dust, potassium sulfate, potassium sulfide, sodium hyposulfite, sulfur, and caustic lime in experiments to control root knot nematode in Florida. He also applied carbon bisulfide to living plants which were killed by the chemical because it is highly phytotoxic. Neal did not obtain satisfactory results with carbon bisulfide probably because he allowed an interval of only one day between treatment of the soil and planting. He did, however, indicate that tobacco dust was the most satisfactory of the treatments he tried.

It is apparent from the literature that until 1940 numerous chemicals were investigated as possible nematocides for the control of nematodes. Chloropicrin appears to have been the only satisfactory chemical tested prior to 1940 for the treatment of nematode infested soils on a commercial scale.

Chloropicrin was first reported to be an effective nematocidal chemical for use in soil by Matthews (1919). Subsequently, Johnson and Godfrey (1932) reported excellent control of root knot nematode in pineapple soils. This chemical is now widely recognized for its nematocidal, fungicidal, and herbicidal properties. As a nematocide, it was once widely used in pineapple soils in Hawaii where satisfactory increases in yield were obtained. Chloropicrin has a fairly high vapor pressure (Table I) and it is most effective when confined to the soil by impervious covers, by a water seal, or by the use of high dosages. The high cost of soil treatments with chloropicrin (approximately $150 to $200 per acre) has tended to restrict its use to crops of high value per acre.

Taylor and McBeth (1941) developed the spot treatment method of applying chloropicrin and this made it economically feasible to treat large acreages of melons where treatment of the planting site reduced the cost to approximately $5 per acre.

Methyl bromide is sometimes used as a soil fumigant to control nematodes. Richardson and Johnson (1935) reported it to be an effective nematocide and subsequent workers have reported the conditions for its most effective use. The chemical has had rather restricted use as a nematocide except for special crops of high value but is very effective in situations where fungicidal and herbicidal qualities are desirable. Methyl bromide has a high vapor pressure and a low boiling point, it can be effectively used only if the vapors are confined to the soil by gas impervious covers. The chemical is highly toxic to man and extreme care must be exercised to avoid breathing the vapors. A small amount of chloropicrin is frequently added to methyl bromide so that it will be more easily detected by persons using it as a fumigant.

TABLE I
PHYSICAL PROPERTIES OF NEMATOCIDES

Nematocide	Boiling point (°C.)	Vapor pressure (25° C.)	Specific gravity	Solubility (gm./100 ml. water)	Physical state
Chloropicrin	112	24.0	1.65	0.20	Liquid
Methyl bromide	45	1824	1.73	0.09	Gas
D-D Mixture	<95–150>	40.4	1.2	0.27	Liquid
Ethylene dibromide	131.6	12.0	2.17	0.43	Liquid
1,2-dibromo-3-chloropropane	196	0.9	2.08	0.15	Liquid
Sodium *N*-methyl dithocarbamate	—	—	—	72.2	Solid
VC-13	124–130 (0.25 mm.)	—	—	0.245 (mg./l.)	Liquid

The era of modern nematocides had its beginning with the discovery by Carter (1943) of the nematocidal properties of 1,3-dichloropropene-1, 2-dichloropropane mixture (D–D mixture). This was the first relatively cheap nematocide that could be used to control nematodes in soil at a cost that permitted large-scale commercial treatments. Unlike chloropicrin and methyl bromide, D–D mixture, because of its lower vapor pressure, does not require the use of impervious covers or water seals. In 1945, Christie reported that ethylene dibromide had given excellent control of root knot nematode in the soil in preliminary tests. Subsequently, this chemical and D–D mixture have been widely used to control nematodes in the soil.

Unlike many of the previous chemicals that were used to control nematodes, D–D mixture and ethylene dibromide have very little effect on soil fungi at the dosage rates ordinarily employed to control nematodes in the field. Both chemicals do, however, kill soil animals and both have been used as soil insecticides. Ethylene dibromide was widely used to control wireworms (Lange, 1947) until the advent of the chlorinated hydrocarbon insecticides.

Since the discovery of D–D mixture and ethylene dibromide, one additional chemical has been developed that is now widely used, this is 1,2 dibromo-3-chloropropane reported by McBeth and Bergeson (1955). Later in 1955 Allen *et al.* reported the chemical to be an effective nematocide based on experiments conducted in 1953 and 1954. This material presents a new aspect, low phytotoxicity. Thus, it can be safely applied to living plants.

Historically, the discovery of D–D mixture and ethylene dibromide and their subsequent wide use as low cost effective nematocides has had a very profound influence on our knowledge of plant pathogenic nematodes. Many nematodes ectoparasitic on roots have been discovered by the use of one or the other of these nematocides in fields where crop growth and yields were reduced from unknown causes. In addition, the use of these materials has made possible a more accurate appraisal of the damage that should be attributable to nematodes. The spectacular increases in yield that have frequently resulted when nematodes are controlled have served to make growers aware of the necessity of employing nematode control procedures. The net result of the use of these materials has been the awakening of a widespread interest in nematodes as plant pathogens, recognition that nematodes are frequently important in certain disease complexes, and renewed activity in the study of nematodes.

IV. The Effect of Nematode Control on the Crop

Nematocides are usually applied in situations where control of nematodes will result in increased quantity or quality of yield of the harvested portion of the crop. (Fig. 1). It is not possible to cite all instances of increased yields that have been reported in the literature. The fact that the use of nematocides has increased many fold during the past 15 years is ample testimony to the effectiveness of nematocides in increasing crop yields. Thorne and Jensen (1946); Raski *et al.* (1953); Allen *et al.* (1955); Lear and Thomason (1956); Stark *et al.* (1944) and numerous other investigators have reported the increased yields resulting from the use of nematocides.

There is no method, aside from experience, that permits an accurate forecast of the yield increases that can be obtained from nematode control treatments. Over a period of 10 years when all plots were carefully selected on the basis of previous nematode damage from root knot nematode and for soil types favorable to nematocidal treatments, the yield of lint cotton in California tests has varied from no significant increase to more than one bale per acre with both broadcast and row placement treatments. The percentage increase does not appear to be correlated with the yield obtained on untreated soils. For example, Allen and Raski (1950) obtained yield increases of 0.6 to 1.0 bale of lint cotton when the yields on untreated plots were only slightly more than one bale per acre. In 1957 preplanting row placement treatments with 9 gallons of D–D mixture per acre resulted in a yield of 3.50 bales of lint cotton per acre on land that produced 2.57 bales of lint cotton without treatment. Although satisfactory yields are sometimes obtained on land which is heavily infested with nematodes, this should not

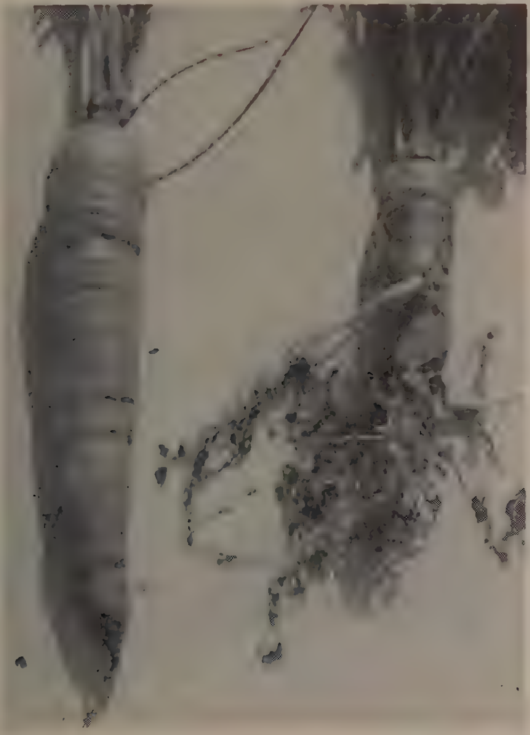

FIG. 1. Left, uninfested carrot; right, carrot infested with root knot nematode.

necessarily be the final criterion in determining the necessity of controlling nematodes. This is so, because of the variety of soils, and cultural practices that are encountered in the field. If it is known that nematodes are present, then exploratory treatments should be undertaken to determine the advisability of applying a nematocide.

Nematocides applied for the express purpose of securing the maximum reduction in populations and maximum yield that are possible are applied as area treatments. In these treatments, an attempt is made to kill all of the nematodes in the upper layers of the soil. Conventional treatments of this type involve the injection of the volatile nematocides by shanks spaced 12 inches apart and to a depth of 8 inches in the soil. D–D mixture at 20 gallons per acre, 83% ethylene dibromide at 4–6 gallons per acre, or 1,2-dibromo-3-chloropropane at 1 to 2 gallons per acre are the dosage rates ordinarily used to control root knot nematode in sandy or sandy loam soils. Because of the extreme range of soil types it is not possible to predict the exact results that can be obtained from a specific treatment. However, the application of 20 gallons of D–D mixture per acre to a cotton field in California in 1946 resulted in nearly 100% control of root knot nematode larvae to a depth of 5 feet. Examination of the roots of cotton grown subsequently in this field also indicated a high degree of control. This treatment satisfactorily controlled the nematodes for two crops of cotton. However, this is an exceptional circumstance as indicated by Allen *et al.* (1955). Row treatments with 9 gallons of D–D mixture per acre increased cotton yields by 0.3 bale of lint cotton per acre on plots treated the previous year with 20 gallons of D–D mixture per acre. More striking results have been obtained in tests on sugar beet nematode by Thorne (1952) where there was complete crop failure of beets planted on soil that had been treated and had grown a crop of beets the previous year.

There are nematocidal treatments that are directed toward eliminating all of the plant pathogenic nematodes in the soil. Such is the case in the preplanting treatments of soils for perennial crops such as citrus, walnut, apple, cherry, peach, grape. Here it is necessary to apply high dosages so that kills are obtained to considerable depths in the soil. However, even in sandy soils kills are only obtained to a depth of about 8 feet.

Row placement treatments are a variation of the spot treatments suggested by Taylor and MacBeth (1941). They are applied to protect the root system of the plant from early attack by large numbers of nematodes. The degree of control is not as high as is obtained with area treatments, and usually the increases in yield are not as great (Raski *et al.*, 1953). However, a lesser amount of the fumigant is applied so that the net profits obtained from row treatments are usually about equal to those from area treatments. The amount of nematocide required for row placement treatments varies with the row spacing. In cotton where row spacings are 38 to 40 inches, satisfactory control of root knot nematode can be obtained by the application of 7 to 9 gallons per acre of the dichloropropene nematocides, 2.5 to 3 gallons per acre of 83% ethylene dibromide or 0.5 to 0.75 gallons per acre of 1,2,dibromo-3-chloropropane. The amount required is less if row spacings are greater than those indicated. Row placement treatments may be applied with one, two, or three chisels spaced at 12 inches depending on the crop to be grown.

A. *Nutritional Effects*

Plants injured by nematode infestations exhibit symptoms that are characteristic of nutritional deficiencies. The poor growth and symptoms of sugar beets infested with sugar beet nematode were once thought to be caused by a potassium deficiency. However, such plants failed to respond to soil applications of potassium, or other fertilizers and it was later found that the primary cause of the trouble was sugar beet nematode (Fig. 2). Nematode infested plants are frequently deficient in certain essential elements and it is not surprising that control of nematodes sometimes results in the correction of deficiency symptoms. One of the corrective measures that is first used by growers on crops that exhibit poor growth and yellowing of the foliage is the application of additional amounts of fertilizer. This procedure has been undertaken with little success in many problems that involved nematodes. When the problem has been corrected by the control of nematodes, the plants grown on treated soil appear to be greatly stimulated and symptoms of nutritional deficiency have been alleviated. This situation has caused

FIG. 2. Sugar beet field showing injury and loss of stand caused by sugar beet nematode.

some investigators to postulate that nematocides applied to the soil cause the release of some essential nutritional elements. Although there does exist the possibility of some ion exchange as the result of the application of nematocides, such claims have not been substantiated. The so-called "increased growth response" of plants grown on soil treated to control nematodes is now recognized to be due largely to the control of nematodes and the subsequent development of normal, functional roots by the plant.

The effect of soil fumigation with D–D mixture and chloropicrin on nitrification and growth of pineapple has been studied by Tam (1945). He found that these nematocides suppress nitrification, that ammonia accumulates in the soil, and that pineapple plants are able to obtain nitrogen from the ammonia. He indicated also that chloropicrin suppresses nitrification for a longer period of time than D–D mixture because of its greater toxicity to nitrifying bacteria. Increased amounts of nitrogen are also present in the leaves of plants grown on treated soil.

Tarjan (1950a) reported that boxwood infested with *Pratylenchus* spp. is deficient in several elements and there was some indication of an increase in the amount of sodium in the leaves. Oteifa (1952) found that lima beans infested with root knot nematode have lower amounts of nitrogen, phosphorus, calcium, magnesium, and potassium. He also indicated that there is differential uptake of these elements in the presence of nematodes. Sher (1958) found that the leaves of roses infested with the root lesion nematode, *Pratylenchus vulnus* Allen and Jensen, 1951, contain lower amounts of iron, copper, and magnesium. The foliage of the infested roses exhibits symptoms of deficiency disease. Rose plants not inoculated with the root lesion nematode and growing in the same soil as inoculated rosese do not show symptoms of deficiency. It might be anticipated that one of the side effects of controlling parasitic nematodes would be restoration of normal nutrition of plants if they are growing in soil with sufficient available amounts of required elements. This seems to be so in the field since plants grown on treated soil are usually normal in appearance and make vigorous growth.

B. *Water Relationships*

That nematode infested plants suffer from water deficiency was observed many years ago in sugar beets infested with sugar beet nematode. Infested plants frequently exhibit severe wilting during the daytime and recover at night. In cases of heavy infestations, this condition of wilting becomes permanent and the plants do not recover regardless of the application of large amounts of irrigation water. This is an extreme example and does not occur to the same extent in other crops.

In conducting trials in California with nematocides to control root knot nematode infestations of cotton we have observed that cotton plants growing on untreated plots show evidence of the need for irrigation in advance of plants on treated plots. In these experiments, if irrigations were applied on the basis of the need of the plants on the untreated plots, then plants on treated plots were irrigated too frequently. This results in a lowered yield on the treated plots due to the increased vegetative growth induced by overirrigation. It has been the experience of growers that the use of nematocides on root knot nematode infested soils frequently allows a reduction in the number of irrigations required when the same soils were not treated for nematode control. It is sometimes stated by growers that nematodes not only reduce yields, but they also increase the production costs because of the excessive amounts of fertilizers and water that are required to grow susceptible crops on infested land.

C. *Disease Complexes*

The associations of nematodes with certain fungal and bacterial diseases of plants have been suggested by a number of early authors. Gilbert (1914) suggested that *Fusarium* wilt of cotton causes greater crop losses when root knot nematode is present. Hastings and Bosher (1938) found that meadow nematode and *Cylindrocarpon radicicola* Wr. cause greater damage in combination than is caused by either organism alone. Young (1939) presented evidence indicating that root knot nematode decreases resistance of many tomato varieties to *Fusarium*. To the present time there have been numerous contributions to the subject of disease complexes involving nematodes and other organisms. Some of the best known of these associations are as follows: meadow nematode and brown root rot of tobacco (Valleau and Johnson, 1947; Jenkins, 1948b); meadow nematode and root rot disease of small grains (Jenkins, 1948a); *Aphelenchoides ritzema-bosi* and *Corynebacterium fascians* in cauliflower disease of strawberry (Crosse and Pitcher, 1952); sting nematode, *Belonolaimus gracilis*, and *Fusarium* wilt of cotton (Holdeman and Graham, 1954); reniform nematode, *Rotylenchulus reniformis*, and *Fusarium* wilt of cotton (Neal, 1954); root knot nematodes, *Meloidogyne* spp., and black shank of tobacco (Sasser *et al.*, 1955); root knot nematode and Granville wilt of cotton (Lucas *et al.*, 1955); nematodes and bacterial wilt of carnations (Stewart and Schindler, 1956); tobacco stunt nematode and *Fusarium* wilt of tobacco (Holdeman, 1956); root knot nematodes, *Pratylenchus* spp., *Tylenchorhynchus claytoni* and black shank of tobacco (Moore *et al.*, 1956); root knot nematode and *Rhizoctonia* disease of cotton (Reynolds and Hanson, 1957).

One of the most significant developments in these disease complexes involving nematodes is the discovery that certain disease-resistant varieties lose their resistance when nematodes are present along with the fungus or bacteria. Equally important is the fact that if nematodes are controlled by nematocides the plants do not lose their resistance. It is also of interest that some of these relationships between nematodes, pathogenic fungi, and bacteria, have been discovered through the use of nematocides. Certain of these diseases have been controlled by the use of nematocides before there was evidence of the cause of the disease. These instances have sometimes resulted in the discovery that nematodes are the primary cause of the disease and that other associated organisms are secondary, e.g., brown root rot of tobacco. Perhaps the most far-reaching of the developments in this field is the discovery that nematodes are sometimes responsible for breaking of resistance and that resistance can be preserved by the use of soil treatments that reduce or control nematode populations.

V. FOLIAR AND SEED-BORNE NEMATODES

Most pathogenic nematodes live in the soil and invade the roots of plants. On that account, most nematocides are soil treatments.

Still, there are a few foliar and seed-borne nematodes and they will be treated briefly before going into the main topic of soil-borne nematodes, and soil nematocides.

A. *Foliar Nematodes*

Several species in the genus *Aphelenchoides* are pathogens on the foliage of plants. They are ordinarily not present in large numbers in the soil, even in soil around infested plants. Control of these species with nematocides involves treatment of the foliage with chemicals that have relatively low phytotoxicity. A unique characteristic of several of these species is their ability to be ecto- or endoparasitic depending on the host plant. *A. ritzema-bosi* (Schwartz, 1911) is endoparasitic in chrysanthemum, but if transferred to strawberry it becomes an ectoparasite. It has been observed to be both endoparasitic and ectoparasitic on gooseberry. *A. fragariae* (Ritzema Bos, 1891) is ectoparasitic on strawberry but becomes an endoparasite in Croft lily and ferns. As ectoparasites these nematodes ordinarily are located in tightly folded leaf and flower buds. When the leaves unfold and are exposed to drying, the nematodes soon disappear from the exposed surfaces.

Practical control of foliar nematodes with chemicals has been obtained with relatively few materials. Chemicals are not ordinarily used to control any of the *Anguina* spp. that attack cultivated plants.

The use of sodium selenate as a systemic nematocide to control foliar nematode infestations in chrysanthemums has been reported by Dimock (1944). Tarjan (1950b) investigated the use of this material as a soil treatment to control *Pratylenchus* spp. infesting boxwoods and indicated that root populations are significantly reduced.

Practical control of foliar type nematodes by the use of chemical sprays became possible with the development of the organic phosphates as insecticides. Raski and Allen (1948) used parathion as a foliar spray on strawberries infested with *Aphelenchoides fragariae.* Populations were reduced to a low level, but satisfactory control was not obtained in tightly folded leaf buds. Dimock and Ford (1950) obtained "spectacular" control of the foliar nematode of chrysanthemum, *Aphelenchoides ritzema-bosi,* with parathion sprays. Systox sprays have been found to be effective in experiments on the stem and bulb nematode in daffodils by Feder (1952) and in alfalfa by Bergeson (1955).

B. *Seed-Borne Nematodes*

Nematodes carried as seed contaminants, particularly stem and bulb nematode on onion, teazle, and clover seed can be successfully controlled by fumigation with methyl bromide, T. Goodey (1945) and J. B. Goodey (1949). However, methyl bromide fumigation is not effective against nematodes imbedded in plant tissues or within seeds or galls. This is undoubtedly due to poor penetration and to the fact that nematodes within galls (wheat nema) are usually very inactive at the time of treatment. 3-*p*-chlorophenyl-5-methyl rhodanine has been reported to control *A. besseyi* infestations of rice seed. Hot water treatments have been much more satisfactory for the treatment of bulbs and corms infested with nematodes, (Chitwood *et al.,* 1941).

VI. SOIL-BORNE NEMATODES

Since most plant pathogenic nematodes live in the soil and attack the plants from that position, our best information deals with soil-borne nematodes. As a basis for understanding how nematocides act and how nematocides are used, we must first discuss the biology of the nematodes themselves.

Plant nematodes, with few exceptions, are unable to complete their life cycle except in the presence of higher plants. Their life cycles are relatively simple and consist of egg, larval, and adult stages. All species, that have been thoroughly studied, have been found to undergo one larval molt in the egg, so that the youngest active stage found in the soil or within plant tissue is usually the second stage larva. There are ordinarily three larval stages outside the egg. These larval stages and the adults are found in the soil in some species of plant parasites while in other species only the second stage larvae are found in the soil. Because of differences in parasitic habit and certain specializations exhibited by some groups of plant pathogenic nematodes it is desirable to classify them into a number of groups and consider each group separately.

A. *Ectoparasites of Roots and Underground Parts of Plants*

The ectoparasitic nematodes are characterized by having all stages present in the soil around the roots of host plants. These nemas feed upon plant cells by means of the stomatostyle or odontostyle depending on the particular parasite concerned. Ordinarily, the ectoparasites of the underground parts of plants do not enter the roots. They are able to move from one feeding site to another and they are active nematodes in the larval and adult stages. These nemas are also characterized by the fact that they lay their eggs singly in the soil and the eggs are not known to be protected by any specialized structures other than the egg shell.

In considering control of ectoparasites of underground plant parts we can conclude that it is unlikely that there will be present, in the soil, in the absence of a host, large numbers of eggs. The majority of the soil population will be adults and larvae. So far as is known these stages show no particular resistance to the action of nematocides. The ectoparasites are regarded as easily killed by present-day nematocides. An exception is the stubby root nematode according to Perry (1953). It has been reported by Todd and Furgeson (1956) that ethylene dibromide is superior to D–D for control of tobacco stunt nematode and by Good and Parham (1957) that ethylene dibromide is more effective against sting nematode.

Ectoparasitic nemas should theoretically be easier to control than endoparasites with postplanting applications of nematocides because they are not protected by plant tissues and because eggs deposited in the soil are without specialized protective structures. Included in this group are nemas in the following genera: *Trichodorus, Tylenchorhynchus, Rotylenchus, Helicotylenchus, Belonolaimus, Dolichodorus, Xiphinema, Longidorus, Criconemoides, Paratylenchus, Hemicycliophora,* and *Criconema.*

B. *Vagrant Endoparasites*

Nematodes in this group are characterized by their endoparasitic habit as well as by the fact that larval and adult stages retain their ability to move actively in soil and roots. The females and males do not, at any time, become sessile or saccate. In some respects the vagrant endoparasites are similar to the ectoparasitic group in that they are able to leave the feeding site. In addition, eggs are deposited singly. Unlike the ectoparasites the vagrant endoparasitic species deposit the majority of their eggs within the host tissue. Thus, it is possible for the individual nematode to spend its entire life span within the host and never be exposed to the soil environment outside the host tissues.

The presence of endoparasitic nematodes within host tissues has an important implication with respect to the application of nematocides to soil. It is well established that nematodes in undecomposed root, bulb, corm, or tuber tissues are protected from the toxic action of the various nematocides. In order to kill the nematodes and their eggs satisfactorily within the plant tissues, greater volumes of fumigant are usually required. It is, therefore, extremely important to recognize the type of nematode involved and to undertake preplanting treatments only after the infested roots of previous crops have decomposed or to use the higher dosage rate.

Species in the genera *Pratylenchus* and *Radopholus* are the most commonly encountered vagrant endoparasites. The nemas in these genera are frequently more difficult to control in ordinary field preplanting treatments than the infective larvae of the root knot nematodes.

There is a marked difference in ectoparasitic and vagrant endoparasitic nematodes with respect to their control with postplanting applications of nematocides. The protection afforded the endoparasites by the host tissues adds materially to the difficulty of securing adequate population reductions. It has been observed that the vagrant endoparasitic forms that are outside root tissues are readily killed by postplanting treatments but those individuals inside plant tissues ordinarily survive treatment unless the root tissues are also killed by the nematocide. It is thought that nematocides with relatively low phytotoxicity and long residual action are more effective because they remain in the soil and are toxic to nemas that leave plant tissues. Experimental results to the present time indicate that postplanting treatments are generally of little value when applied to heavily infected mature plants.

C. *Exposed Endoparasites (Noncyst-Forming)*

The placement of several forms in this classification is questionable, but they do differ in their parasitic habit from those nemas previously discussed as well as those discussed in the following sections. These nemas undergo rather extreme morphological changes in their development. The females become saccate and are unable to move from the site of feeding after they have penetrated into host plant tissues. The exposed sessile endoparasites never completely penetrate into the host tissue. The infective stage penetrates only partly into the host with the posterior portion of the body remaining outside the root. Males of these species do not penetrate plant tissues .

In addition to their sessile habit these nemas deposit their eggs into a gelatinous matrix which serves as a protective device against drying and possibly affords some protection from parasitic and predaceous soil organisms. It is also known that the gelatinous matrix affords some protection from the toxic action of nematocides. However, unlike the nemas in the previous group the exposed sessile endoparasites are not at any time completely protected by plant tissues, and are exposed to the toxic action of nematocides regardless of whether they are present on roots at the time of treatment. They are generally regarded as more susceptible to the action of nematocides than those nematodes that are completely protected by root tissues. The major characteristic of this group is the presence of the gelatinous matrix and the production of a relatively large number of eggs. At certain times most of the population of nemas in this group occur as eggs. Unsatisfactory results may be obtained under such conditions unless the nematocide used has high ovicidal activity.

The two best known species in this group are the citrus nematode, *Tylenchulus semipenetrans* Cobb, 1913, and the reniform nematode, *Rotylenchus reniformis* Linford and Oliveira, 1940. Some reports indicate that postplanting treatments of crops infested with these nematodes have been more successful than of crops infested with endoparasites that are completely embedded in root tissues. Experimental results with such treatments on citrus infested with citrus nematode have been promising using 1,2-dibromo-3-chloropropane. This material and ethylene dibromide have been used successfully as side dressing treatments on pineapple infested with reniform nematode. In the latter case the side dressing treatments are made at intervals following planting of the crop on beds receiving preplanting treatments with D–D mixture or ethylene dibromide.

D. *Nonexposed Sessile Parasites*

In this group are included the root knot nematodes, *Nacobbus* species, and *Ditylenchus radicicola* (Greef, 1872), a pathogen on grasses. These nematodes are characterized by complete penetration of the infective stage into the host plant. In many instances the nematodes mature and produce eggs that are wholly or partly embedded in plant tissue. In other cases, e.g., root knot nematodes, the female is inside the plant tissue and the eggs are deposited in a gelatinous matrix that is wholly or partly outside the tissue. Additionally, species in these genera cause the formation of galls which completely or partially enclose the nematode (Fig. 3). Under ordinary circumstances control of these nematodes is directed against the infective stage or stages in the soil. The second stage larva is the infective stage of root knot nematode and this probably is also the case with *Nacobbus*. Abundant experimental evidence in the literature indicates that when eggs and larvae occur in the roots, satisfactory control is difficult to obtain. McClintock (1915) subjected egg masses of root knot nematode to mercuric chloride, formaldehyde, nicotine sulfate, lime sulfur, and sulfuric acid and concluded that nematodes in egg masses are exceedingly difficult to kill. It has been shown by Taylor (1943) and Stark (1948) that the presence of undecomposed roots containing egg masses presents one of the most difficult problems in controlling root knot nematode.

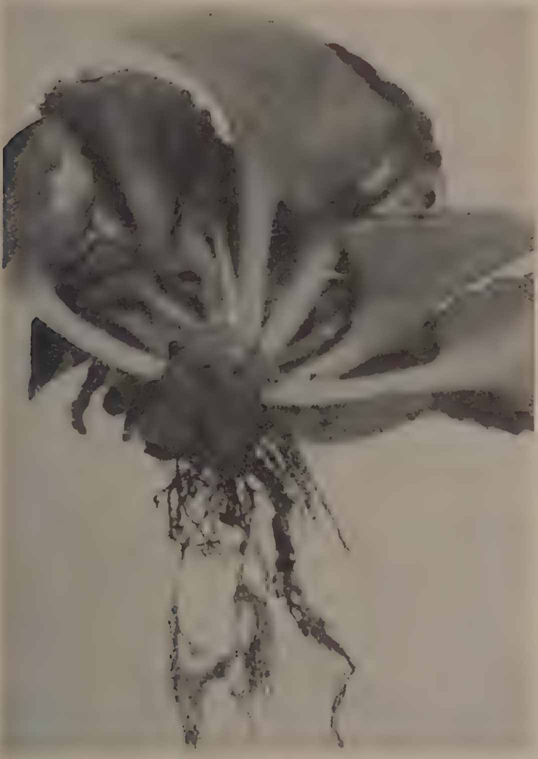

FIG. 3. African violet infested with root knot nematode and showing large multiple galls.

The difficulties of killing nematodes in tissue and eggs protected by the gelatinous matrix apply when control of root knot nematodes is attempted around the roots of living plants. The infective larvae in the soil are readily killed by the nematocide. In most cases the soil populations build up to a high level within a short time following treatment because of the numbers of nematodes escaping the action of the nematocide. Satisfactory control under such conditions has not been obtained with regularity in experimental plots. The nematocidal chemicals that are presently available for use around the roots of living plants are not satisfactory in all respects. In order to control nematodes adequately by treatment of living perennial plants, it would be desirable to have a nonphytotoxic systemic nematocide or a nonphytotoxic nematocide having long persistence in the soil.

E. *Cyst-Forming Nematodes*

The nematodes in this group belong to the genus *Heterodera*. They differ markedly from the preceding groups in their biology because of the presence of the cyst stage. The infective larvae completely penetrate into host roots, but as the body of the immature female enlarges, it bursts from the root tissue, but remains attached to the feeding site by the head and neck. The female body becomes saclike and completely filled with eggs at maturity. The nematode then gradually changes color from thickening and tanning of the body wall, ceases to feed, and may become detached from the root.

The female body filled with eggs is referred to as the cyst. There are actually two types of biologies within this genus. Some *Heterodera* spp. produce a gelatinous matrix into which the female deposits some eggs immediately upon reaching maturity. However, the majority of eggs are retained within the body. This type of biology is characteristic of *H. schachtii* Schmidt, 1871, *H. cruciferae* Franklin, 1945, *H. trifolii* Goffart, 1932, and *H. glycines* Ichinohe, 1952. The eggs that are deposited in the matrix hatch within a short time and the larvae are infective. In all of these species there may be several generations during the season. This is the result, in part, of the hatching of eggs deposited in the gelatinous matrix and of eggs that hatch from the cyst during the same season they are produced.

In some species of *Heterodera* the females do not produce a gelatinous matrix. All of the eggs are retained within the body. These eggs ordinarily do not hatch except in the presence of a hatching factor produced by the roots of certain plants. This means that the major portion of the soil population remains as eggs within the cyst except in the presence of plants producing the hatching factor. *H. rostochiensis* Woll., 1923, the golden nematode of potato, is the best known example of this type of biology.

Allen and Raski (1950) have reported that eggs within the cysts of *H. schachtii* are about as susceptible to D–D mixture as the infective larval stages of root knot nematodes. Mai and Lautz (1953) found the larvae of *H. rostochiensis* to be slightly more susceptible than eggs in cysts. However, satisfactory control of field populations of *Heterodera* spp. are much more difficult to obtain than is the case with *Meloidogyne* spp. Thorne and Jensen (1946) indicate that in Utah and Idaho satisfactory control of *H. schachtii* can be obtained in most instances with 25 gallons of D–D mixture per acre. In other areas, due possibly to other soil factors, satisfactory controls are not regularly obtained. The cyst-forming nematodes present a difficult control problem with nematocides because each cyst represents a discrete colony of nematodes. An important reservoir of infection remains if the nematocide fails to kill the eggs within a single cyst since each cyst may contain several hundred eggs. Most large-scale field treatments are designed to reduce nematode populations to levels that enable the production of satisfactory yields. It appears that the cyst colonies are to some extent responsible for the failure of nematocides to reduce soil populations except when dosage rates are increased, sometimes beyond economic levels.

F. *Nematode Distribution in the Soil*

One of the main obstacles preventing complete eradication of nematodes is their distribution in the soil. It is now generally recognized that most nematode infestations occur throughout the root zone of the particular crop. To kill all the nematodes present in the soil requires treatment with nematocides that will diffuse or be carried by water to depths of several feet. Root knot populations are greater at depths of 3 to 4 feet than at 1 to 2 feet in many California fields. Under such circumstances commercial control is obtained by killing most of the nematodes in the top 3 feet of soil. However, with perennial crops, e.g., citrus, grapes, and deciduous trees it is frequently necessary to treat with dosage rates that kill nematodes to a depth of at least 8 feet. Kills at such depth can be obtained only by using large amounts of toxicant under proper conditions for the best nematocidal action.

The upper 2 or 3 inches of soil frequently contain living nematodes following fumigation with volatile liquid nematocides such as D–D mixture and ethylene dibromide. This is primarily due to the rapid diffusion of these nematocides into the atmosphere from the surface layer. This rapid loss prevents the attaining of lethal concentrations in the surface layers. The presence of a few nematodes at the surface after treatment is not important with most annual crops. However, they are a problem in nursery crops subject to quarintine regulations and in treatments designed to give the long protection required with the tree and vine crops. Gas-tight covers, water seals, high dosage rates, and split applications can be used if maximum kills are required. Vapam has been reported by Baines *et al.* (1957b) to be very efficient in killing all nematodes to depths of 5 or 6 feet when applied in water drenches.

The presence of compaction layers, plow soles, and large clods can protect nematodes under some circumstances. Thorne (1939) has shown that a plow sole restricts the downward dispersion of calcium cyanide applied for sugar beet nematode control.

VII. Methods of Applying Soil Nematocides

The fact that most successful nematocides are volatile liquids with relatively low boiling points, and with few exceptions rather high vapor pressures, has necessitated the development of special types of application equipment. For the treatment of relatively small areas a number of kinds of hand applicators (Fig. 4) have been devised.

A. *Pressure Applicators*

Many applicators operate on the principle of a displacement pump and are designed to deliver accurate dosage rates as well as different dosage rates depending on the length of the stroke. Taylor (1939b) developed and was granted a public patent for such an applicator. All subsequent applicators employ the same principle although numerous improvements have been devised. Hand applicators are designed to deliver a measured amount of nematocide into the soil at the injection site. The depth of the injection is governed by the use of probes of varying length (Fig. 4). The spacing of the injection sites may be varied depending on soil type, dosage rate, volatility of the nematocide, etc. In using hand applicators it is customary to mark the soil surface so as to properly space the injections. Taylor (1939a) and Chitwood (1939) have described methods of determining the most efficient spacings for the injection of nematocides in single injection sites. The method depends upon a knowledge of the effective lateral diffusion of the nematocide in a particular soil type. This is determined by a bioassay method involving nematode kills obtained at varying distances from the injection point. The entire procedure is aimed at securing the most economical dosage rate and spacing for satisfactory nematode control.

In the treatment of large areas the principle of continuous flow is employed rather than injection of the nematocide at a single point. Virtually all of such equipment in use at the present time employs the principle of continuous flow through a tube mounted on a chisel or shank that is drawn through the soil by power equipment (Fig. 5).

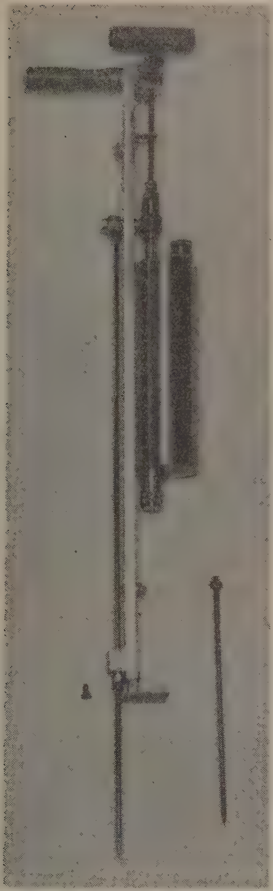

FIG. 4. Hand applicator for injecting volatile fumigants into the soil, probes of two lengths are shown.

Essentially the same principle is employed when carbon bisulfide is applied to the furrow in front of a plow share. The applicator devised by Neller and Allison (1935) for introducing chloropicrin and carbon bisulfide into the soil has been modified by growers, equipment manufacturers, and commercial companies. However, nearly all presently used applicators are based on the same principle. Neller and Allison (1935) applied constant pressure to the tank containing the fumigant and regulated the flow of the toxicant by the use of spray orifices and the speed of the machine. Recent modifications include multiple shanks,

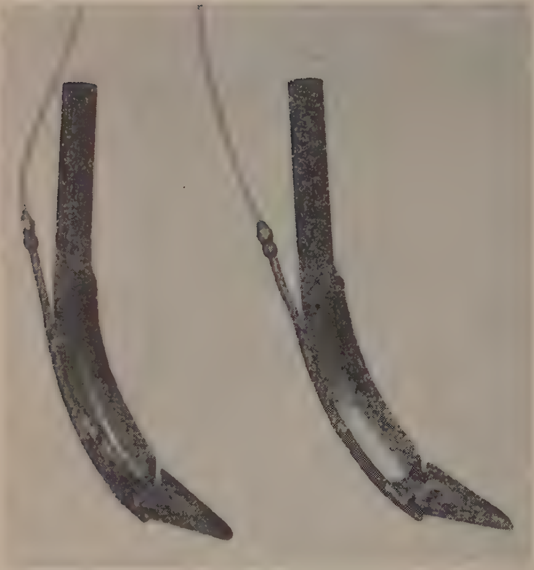

FIG. 5. Chisel shanks for applying volatile soil fumigants in continuous injection lines.

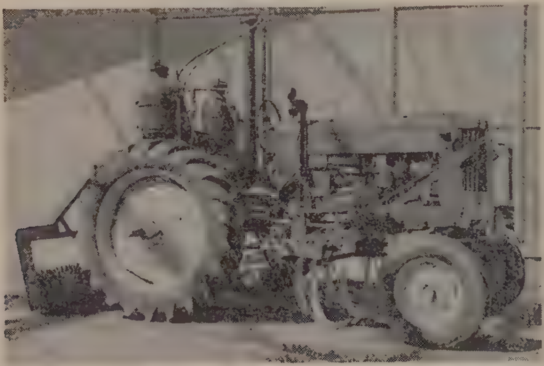

FIG. 6. Fumigant applicator designed for treating experimental plots, showing ring-roller, fumigant containers, and staggered chisels. (Photograph by Bert Lear, Dept. of Nematology, University of California, Davis.)

constant pressure supplied by gear pumps operating from a power take-off, or a ground wheel (Fig. 6). Dosage rates are regulated by pressure, size of orifice, and speed of the machine. Such equipment is usually mounted on a tractor capable of pulling as many as twelve or more shanks through the soil. Various refinements have been devised until it is now possible to accurately apply dosage rates as low as 4 to 6 gallons per acre when the chisel points are spaced at 12 inches so that every square foot receives the desired dosage rate.

B. *Gravity Flow Applicators*

Numerous types of gravity flow applicators have been devised. They are ordinarily employed to treat small acreages since they are relatively trouble free and are obtained at relatively low cost. Gravity flow combined with a constant head in the fumigant container allows for accurate metering of the fumigant. This principle is frequently employed when nematocides are applied with a plow applicator. In this case the nematocide is allowed to flow into the furrow immediately in front of the plow share. Such equipment can be used on both single and multiple bottom plows.

A modification of the gravity flow applicator in which capillary tubing is used in place of the ordinary valve or orifice has made it possible to apply dosage rates as low as 0.5 gallons of liquid nematocide per acre. In this case the rate of flow is governed by the bore of the capillary tube, its length, and the height of the liquid column. The use of this type of applicator depends on a constant speed in order to apply accurate dosages.

C. *Solid Carriers*

The effectiveness of modern nematocides when impregnated upon solid carriers has been reported by Taylor and Golden (1954) and Sasser and Nusbaum (1954). The latter authors applied ethylene dibromide with vermiculite as a carrier and employed a fertilizer rig for applying the impregnated vermiculite. The principle involved is essentially that used in the conventional liquid applicators, that of applying continuous lines of the nematocide to the required depth in the soil. This method has no advantage for field scale applications other than the possibility of using equipment designed to make fertilizer applications. Field tests conducted in California in 1955, 1956, and 1957 indicate that applications of dibromo-chloropropane impregnated on Attaclay were as effective as similar dosage rates of the liquid fumigant. Greater cost per unit volume of nematocide and the difficulty of obtaining accurate dosage rates compare unfavorably with the liquid applications. Equipment designed especially for distributing desired dosage rates of nematocides on solid carriers is not presently available for large-scale treatments. The packaging of volatile fumigants impregnated on solid carriers also adds to the expense of such treatments. Moreover, the nematocide is rapidly lost if the containers are left open for any length of time.

D. *Drenches*

The chemical and physical properties of several nematocides preclude their being injected into the soil. These chemicals are characterized by having extremely low vapor pressures and by not diffusing readily through the soil. Some are relatively insoluble in water and must be applied as emulsions. One currently used material, sodium-*N*-methyl dithiocarbamate (Vapam) is very soluble in water and is ordinarily applied as a water solution drenched into the soil. Drenches containing ethylene dibromide, D–D mixture, allyl alcohol, and similar materials in emulsified form have been used under special circumstances to control nematodes in the surface, 2 to 3 inches of soil. The use of nematocides whose movement in the soil is dependent upon water has been generally restricted to special uses such as preplanting treatments for trees, vines, and nursery beds. Their use may depend on the presence of pathogenic organisms such as fungi, although nematodes may also be present and controlled by the treatment.

E. *Over-all and Strip Treatments*

Soil treatments may be applied over the whole of the area or in narrow strips where the row of crops will be. Area, over-all, or broadcast treatments involve treating the entire area to be planted. The nematocide is usually applied in continuous lines at the desired depth and spacing so as to effectively treat as much of the soil as possible. Such applications may also be made by hand injections. Conventional treatments are usually made by applying the volatile liquid nematocides to a depth of 8 inches with 12 inch spacing of the chisel shanks. For special problems the depth of application and the spacing may be varied. Baines *et al.* (1957a) recommend a spacing of 18 to 20 inches and a depth of 10 to 14 inches for preplanting treatment of old citrus soil. If, however, the soil contains large amounts of clay these authors recommend a spacing of 12 to 15 inches. These treatments involve the application of 50 to 240 gallons of D–D mixture per acre, the amount used depending upon soil type and the depth of the soil.

Row or strip treatments are designed to reduce or eliminate nematode populations in the immediate planting area. Row treatments have been developed over a long period of time and for a variety of situations. The treating of planting sites with carbon bisulfide for the control of *Armillaria mellea* embodies the same principles. Among the first and most widely used of the row or strip treatments is that used in pineapple fields in Hawaii. Chloropicrin was used in this manner prior to the discovery of D–D mixture. The procedure in this case involved the injection of chloropicrin into the immediate soil area upon which plants were to be grown. The planting area was covered subsequent to injection of the chemical by specially treated paper to aid in retaining the fumigant in the soil. The area between beds was left untreated. D–D mixture has been used in the same manner since it became available. The basic idea is to provide protection for young plants for a period of time that will allow them to become well established before they are subjected to attack by large populations of nematodes.

The protection afforded by row or bed treatments is not equal to that obtained by area treatments, but row or bed treatments are frequently more economical in terms of net profit. It is common practice to employ row or bed treatments to control nematodes on cotton, tomato, bean, melons, and similar crops. Dosage rates are dependent upon row or bed spacing, soil type, the nematocide used, and the susceptibility of the particular crop to nematode damage. Generally row treatments are not employed when the marketable portion of the crop is subject to nematode attack, e.g., carrots, Irish potato (Fig. 1). Row treatments are not employed to treat land that is to be used to grow perennial crops.

F. *Special Procedures*

There has been limited use of nematocides to control *Ditylenchus* spp. Adults and larvae are usually found in the upper layer of the soil. This is the area where most volatile nematocides are least effective and high dosage rates in addition to special procedures are required to secure adequate control which involves covering the soil with gas-impervious covers or using split treatments. The latter procedure is carried out by applying one-half the dosage rate and then plowing after a 2 or 3 day interval and injecting the other half. Dallimore (1955) has reported that a scraper attachment mounted in front of the plow provides a successful means of adequately treating the upper 3 or 4 inches of the soil. The nematocide is injected and after an interval of 4 to 5 days the soil is plowed, at which time the scraper pushes the top soil into the furrow. The plow then covers the soil in the furrow where it is exposed to the action of the nematocide. This method is expressly for the purpose of treating the upper layers of the soil and seems to be effective only against such nematodes as *Ditylenchous dipsaci* (Kuhn, 1857) and *D. destructor* (Thorne, 1945) that are found in upper soil layers. The method has no appreciable advantage for use against species that are distributed to considerable depths in the soil, e.g., root knot nematodes, cyst nematodes, burrowing nematode, and root lesion nematodes.

VIII. Methods of Confining Soil Fumigants

The effectiveness of volatile nematocides as a means of controlling nematodes in the soil is to a large extent dependent upon the retention of toxic concentrations of the vapors in the soil. Certain of the physical properties of the soil, e.g., porosity, clay content, water content, compaction, and organic content, influence retention of the vapors. If gas-impervious covers or water seals are not used to insure the retention of toxic concentrations of the toxicant, then the actual manipulations of the soil and its condition at and subsequent to treatment are extremely important. Various devices have been used to smooth the surface of the soil following the injection of nematocides with chisel-type applicators. Harrowing, dragging a chain, heavy iron bar, or wooden drag behind the applicator tends to seal the soil surface resulting in better retention of toxic vapors (Fig. 7). In most situations, more effective retention seems to result if a ring-roller or similar implement is pulled immediately after the applicator (Fig. 6). In addition to smoothing the surface, the ring-roller has the added advantage of compacting the surface layer. Consistently good results have been obtained in California using this method on experimental plots.

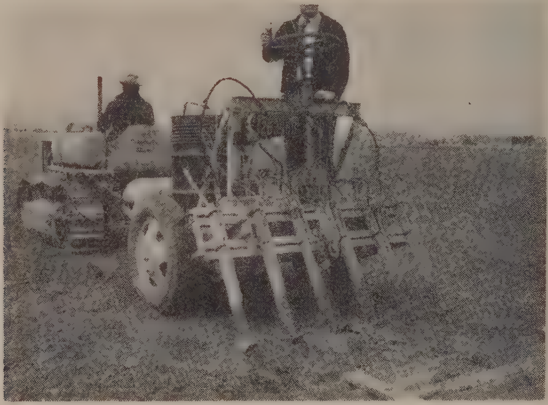

FIG. 7. Early model fumigant applicator mounted on trailer, pulled by tractor. (Photograph by W. H. Lange, University of California, Davis.)

Water seals and gas-impervious covers have been mentioned previously and their main effect is to prevent rapid loss of the nematocide from the soil. Polyethylene covers are regularly employed when soil is treated with methyl bromide. They may also be used with chloropicrin when a high degree of control is desirable. Similarly, water seals increase the effectiveness of chloropicrin. It has been observed in the field that rain immediately following injection of such materials as D–D mixture and ethylene dibromide results in longer retention of the gases with attendant increased control of nematodes. Phytotoxicity to subsequent plantings has also been observed when the interval between treating and planting was not increased to allow more time for the diffusion of the toxicant from the soil.

IX. Treatment of Living Plants (Postplanting)

The injection of volatile liquids into soil around the roots of living plants has been attempted with a variety of materials. D–D mixture and ethylene dibromide are not satisfactory because they are phytotoxic. Of the presently used volatile liquid nematocides only 1,2,-dibromo-3-chloropropane seems to possess low enough phytotoxicity to enable its use around the roots of living plants at dosage rates that are nematocidal. Lownsbery and Sher (1958) have reported significant increase in growth of young walnut trees side-dressed with this material when the trees were planted in soil previously treated with a nematocide. They indicate that growth rates were not satisfactory for trees growing on preplanting fumigated soils, but that subsequent side-dressing treatments increased growth and reduced nematode populations. The use of this material for the purpose of side-dressing established plantings is still in the experimental stage. It is probable that some of the unsatisfactory results that have been obtained are associated with attempts to rejuvenate older plants that had extremely poor root systems.

Satisfactory results have been reported from some experiments using dibromochloropropane to side-dress such crops as cotton and beans for the control of root knot nematode. However, about an equal number of failures have also been encountered in the field. In situations where increased yields have resulted, the increases do not compare favorably with those obtained with preplanting treatments with equal amounts of the same material. In some instances cotton has been severely injured by side-dressing treatments, the treatments also resulted in lower yields than were obtained from untreated areas.

The organic phosphate, *o*-2,4-dichlorophenyl-*o,o*-diethylphosphorothioate, (V–C 13) has been reported to be a satisfactory chemical for treatment of living plants. It is used primarily as an emulsion and drenched into the soil. It is reported to be chemically stable in the soil, has a very low vapor pressure, and its movement in the soil depends on the movement of the water emulsion. Christie and Perry (1958) have discussed various aspects of its use and indicate that it is an extremely promising material for use around living plants. V–C 13 applied as a drench is reported to improve the growth of turf by controlling associated pathogenic nematodes.

Tarjan (1954, 1955) has reported that aqueous emulsions of 3-*p*-chlorophenyl-5-methyl rhodanine completely control root knot nematode infections when applied to potted tomato plants. He reported that the chemical reduces nematode populations in the soil around the roots of strawberry plants when it is mixed in the dry form with the soil.

X. Physical Factors Influencing the Efficiency of Nematocides

The soil is the greatest barrier to the successful use of nematocides. Over a period of years, a large body of evidence accumulated indicates that the success of conventional fumigation treatments is largely governed by the soil to which they are applied. It is also recognized that little can be done to change those characteristics of soils that are unfavorable to the effective use of nematocides. The amount of clay and/or the amount of organic matter are the limiting factors that are fairly well fixed and the difficulties inherent in their presence respond only slightly to any practice that can be reasonably carried out in the field.

It is unfortunate from the practical aspects of killing nematodes, under field conditions, that most of the precise studies on the characteristics of soils that inhibit the diffusion of gases have been made on dry soil. However, there can be no doubt that some of the soil factors discussed in the following sections are operative, although in several instances experimental evidence is lacking or incomplete concerning the mechanisms that influence or restrict diffusion.

A. *Sandy Soils*

Sandy soils and sandy loam soils have relatively low organic and clay contents and are representative of soils in which volatile nematocides as well as other types of nematocides are most effective in controlling nematodes. The effectiveness of volatile nematocides in these soils is directly attributable to the relatively unrestricted diffusion of the toxic vapors. It is known from the work of Hagan (1941), Call (1957), Wade (1954, 1955), and Stark (1948) that carbon bisulfide, ethylene dibromide, and chloropicrin are highly sorbed when they are in contact with dry soil. Hagan (1941) has indicated that soil moisture is the most important factor limiting diffusion. Call (1957) and Wade (1955) have shown that ethylene dibromide sorbed by dry soil can be released by the addition of moisture. It, therefore, follows that moisture must be present in order to prevent the excessive sorption that takes place in dry soil. Lear (1956) has shown that Vapam applied as a concentrate solution to surface soil is retained in the surface of wet soil and is carried out of the surface of dry soil by addition of water. Most consistent results are obtained by applying the complete treatment as a drench.

In the field, volatile nematocides are applied to moist soils. It has been observed that these nematocides are most effective when they are applied to sandy loam soils that are at or near their moisture-holding capacity. We must, therefore, assume that effective treatment of such soils requires a balance between water content and pore space that enables the gaseous phase of the nematocide to diffuse and reach toxic concentrations throughout the soil. It is further assumed that the toxic vapors go into solution in the water phase and nematodes are killed by contact with water solutions.

B. *Clay Soils and Organic Soils*

There are no precise data to indicate the exact clay or organic content of the soil that will restrict diffusion to the point where effective control is not obtained. If we assume as Call (1954) and Wade (1955) have indicated for ethylene dibromide that water inhibits adsorption of this material on soil particles, we must look for other causes for poor diffusion of the gaseous phase of volatile nematocides. As indicated in Table I, the volatile nematocides are slightly soluble in water, varying from 0.09 gm. per 100 ml. of water for methyl bromide to 0.43 gm. per 100 ml. of water for ethylene dibromide. If a conventional dosage rate of 20 gallons per acre of D–D mixture is applied as a broadcast or area treatment, each cubic foot of soil receives a dosage rate of 1.87 ml. of the nematocide. Under field conditions there is sufficient water in each cubic foot of soil to dissolve the entire amount of D–D mixture (sol. 0.27 gm. per 100 ml. of water) applied. It seems likely that in clay and organic soils, having field capacities exceeding 25%, large amounts of the gaseous phase of the fumigant are dissolved in the water surrounding soil particles at the point of injection, thus restricting the degree and speed of diffusion. In addition, the presence of smaller pore spaces in clay soils and the fact that adsorbed water further decreases the size of the pores leads one to the conclusion that clay soils are not successfully treated with volatile nematocides because of the restrictions on movement imposed by water present in these soils. It is highly probable that other factors are operative in the different types of clays and in organic soils where the situation is complicated by factors that are not well understood at present. Goring and Youngson (1957) have shown that diffusion of ethylene dibromide is restricted by the addition of organic material to sandy soil and that different materials have different effects.

In treating clay and organic soils, poor or limited diffusion can be overcome to some extent by the application of higher dosage rates. However, there are economic limitations that sometimes make it impractical to apply dosage rates that are effective. This is clearly the case with sugar beet nematode in clay soils (moisture-holding capacity 20% or above) and in highly organic soils as shown by Allen and Raski (1950). Similarly, the change in dosage rate that is required for various types of soil is indicated by the recommendation of Baines *et al.* (1957a) of 60 gallons per acre of D–D mixture as a preplanting treatment to control citrus nematode on sandy soil and 250 gallons per acre to achieve comparable kills in clay soils.

C. *Moisture*

As indicated in the preceding section, it is impossible to discuss soil type without reference to moisture unless dry soils are being considered. However, the practical use of volatile nematocides never involves completely dry soils. We must, therefore, consider the conditions of moisture that are generally thought to be most advantageous for the use of volatile nematocides. It is obviously not possible to have precise data for every soil type encountered in the field. In sandy and sandy loam soils (moisture equivalent 5 to 12%), best results are obtained when treatments are made at or near field capacity. Soils with high clay content cannot be cultivated when at field capacity and it is necessary that these soils be treated at a time when they are below their field capacity. It is recommended that these soils should be treated when they are in suitable condition for cultural operations and in seed-bed condition so far as tilth is concerned. Van den Brande *et al.* (1954) have indicated that adequate soil moisture must be present in order to effectively reduce *Heterodera rostochiensis* populations. Practically no data are available to make possible a comparison between dry and wet soils. There can be no doubt that reasonable soil moisture is desirable but it is not known whether soils with high clay content could be treated successfully if moisture content is near the wilting point. It is likely that the phenomenon of adsorption by soil particles becomes operative if the moisture content is reduced below this level.

D. *Temperature*

The proper temperature for the application of nematocides is a controversial subject probably because no single investigation has been undertaken that includes factors that are operative under field conditions. McClellan *et al.* (1949) have shown that such materials as chloropicrin, D–D mixture, and ethylene dibromide are more effective at 85 to 95° F. than at lower temperatures. However, these experiments do not have application to field situations since the containers prevented escape of the nematocide. Furthermore, the length of the exposure period was very short so that at lower temperature not only would one expect slower diffusion, but also the activity of the nematodes would be reduced. Although laboratory data may indicate greater nematocidal activity at higher temperatures, there is also more rapid diffusion from the soil into the atmosphere.

Successful nematode control has been obtained by the application of some nematocides to field soils at temperatures as low as 37° F. at the 8 inch depth. It appears that if all other conditions are favorable, there is considerable latitude in the temperature requirements of nematocides. This is a fortunate circumstance since treatments must sometimes of necessity be applied in spring and fall at temperatures that are sometimes lower than those recommended on the basis of laboratory experiments. Van den Brande *et al.* (1954) have shown that treatments made at 36.5° F. are as effective as those made at 59° F.

Although diffusion may be restricted at low temperatures, escape from the soil is likewise restricted so that satisfactory control is obtained, but the period of time to achieve such control is necessarily longer. Goring and Youngson (1957) have shown that ethylene dibromide does not diffuse in soil at 50° F. but they indicate that the nematocide remains in the soil and kills as the temperature rises.

E. *Preparation of the Soil*

Aside from adjusting soil moisture to the optimum level and waiting for favorable temperatures, it is possible to improve the conditions of the soil by cultural operations. Diffusion and resultant kill of nematodes is enhanced when the soil is in a friable condition. Absence of large clods, compaction, and rough surface tend to provide conditions conducive to obtaining maximum efficiency of volatile soil fumigants. Undecomposed crop residues regardless of whether or not they contain nematodes or nematode eggs are detrimental. These residues tend to collect upon the chisels and to make the surface of the soil rough, which in turn exposes more soil surface and increases diffusion into the atmosphere. Stems or roots that protrude from the surface of the soil act as chimneys for the escape of the gaseous phase of the fumigant. Excellent results with the volatile soil fumigants are usually obtained when the soil is properly prepared and is free of crop residues, large clods, the surface smooth, and the moisture content is at a favorable level.

F. *Posttreatment Conditions*

Atmospheric conditions at, during, and following the application of volatile soil fumigants are very important in determining the ultimate effectiveness of a nematocidal treatment. Immediately following treatment the soil surface should be smoothed to fill any channels that may be left by the chisels as they are pulled through the soil. It is preferable to pull a ring-roller (Fig. 6) behind the fumigant applicator. Although wind and temperatures following treatment cannot be controlled, it is important that the soil mass should remain undisturbed for a period of time following fumigation. Any disturbance of the soil surface is apt to increase diffusion from the soil.

If rain immediately follows treatment it will tend to seal the soil surface and increase the efficiency of the treatment. If highly phytotoxic materials such as D–D mixture and similar materials are used, and rain follows the treatment, there is a possibility that retention of the toxicant may result in phytotoxicity if planting follows at the recommended interval. Under such conditions the interval between treatment and planting should be extended. This is extremely important where recommended intervals are based on minimum times under optimum conditions.

XI. The Mode of Action of Nematocides

Practically no information is available concerning the mode of action of any nematocide. Nearly all of these chemicals are biocides and do not show enough specificity to indicate a mode of action that would be peculiar to nematodes. It is assumed that since the phosphate insecticides are toxic to nematodes, these materials act upon the nervous system and the enzyme cholinesterase. It has been suggested by Chitwood and Chitwood (1950) that effective nematocides must be materials that change the permeability of the cuticle and should also be soluble in or dissolved by fat solvents. However, there are little or no data available to support the necessity of penetration through the cuticle. It is easily observed when nematodes are exposed to water solutions of vital stains that penetration is most rapid through the oral opening, phasmids, vulva, and anus. There is no reason to assume that most nematocidal chemicals do not enter the body of the nematode through these avenues as well as through the cuticle. It is reasonable to assume that chemicals that are ovicidal must penetrate through the egg shell in order to be effective. Ethylene dibromide which is not as ovicidal as D–D mixture or chloropicrin does not kill as rapidly as the latter materials. This might indicate that penetration through the cuticle or the egg shell is not as rapid with ethylene dibromide as with the other materials. Data are not available concerning the ovicidal properties of 1,2,-dibromo-3-chloropropane but since it does not kill larvae of root knot nematode as rapidly as ethylene dibromide it can be assumed that this nematocide might also have rather poor ovicidal properties.

The gelatinous matrix produced by root knot nematodes, citrus nematode, reniform nematode, and some cyst nematodes protects eggs from nematocidal action. This has been shown when treatments of living roots are attempted. It has been mentioned previously that the cyst wall does not afford an effective barrier to some nematocides and failure to control *Heterodera* spp. is probably not due to this factor.

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